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Ischaemic Postconditioning in Normal and Type 2 Diabetic Rat Hearts

Thesis submitted by

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ABSTRACT

Background- Coronary heart disease is the leading cause of death in the Western world and targeting those strategies which limit the damage sustained as a result of a lethal myocardial infarction has been a major goal for many years. Two such strategies, ischaemic **pre**conditioning and ischaemic **post**conditioning are the most powerful endogenous cardio-protective phenomena known to man. The mechanisms involved in the newly described phenomenon of ischaemic postconditioning are not fully known. Additionally, the vast majority of studies investigating ischaemic preconditioning have been performed in normal hearts, leading to the suggestion that preconditioning is a "healthy heart" phenomenon. However, conflicting evidence exists as to whether the diabetic myocardium can be protected by ischaemic preconditioning (IPC). This thesis examines the mechanisms involved in these strategies of cardioprotection: (1) ischaemic postconditioning in normal hearts, and (2) ischaemic pre- and postconditioning in type II diabetic hearts.

Methods and Results- Using a Langendorff isolated rat heart model we demonstrated that ischaemic postconditioning significantly reduced myocardial infarct size in normal rat hearts, and that this effect was comparable to that of ischaemic preconditioning. Western blot analysis demonstrated for the first time that postconditioning-induced protection is mediated via the PI3K-Akt pro-survival signalling cascade and its downstream targets, namely, eNOS and p70S6K. However, we found that type II diabetic rat hearts could not be protected using the same postconditioning protocol as a result of insufficient Akt

phosphorylation. Conversely, the type II diabetic myocardium can be protected by ischaemic preconditioning but the threshold required to achieve this protection is elevated compared to non-diabetic hearts. This elevation in threshold is required to achieve sufficient phosphorylation of Akt, to execute the protective signal induced by ischaemic preconditioning.

Conclusion- Our study demonstrates that in normal hearts, ischaemic postconditioning is a powerful strategy for myocardial protection and is mediated via the PI3K-Akt pro-survival signalling cascade. However, presumably due to differences in cellular signalling physiology, ischaemic postconditioning does not have a similar effect in type II diabetic hearts, and the beneficial effect of ischaemic preconditioning is only seen if the preconditioning stimulus is increased sufficiently to achieve enough Akt phosphorylation to mediate the protective signal. This suggests that the human diabetic population may be more resistant to the protective effects of IPC, but that provided the preconditioning stimulus is sufficient, the diabetic myocardium can be protected.

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LIST OF PUBLICATIONS

The following is a list of manuscripts arising from the thesis.

Peer Reviewed Publications:

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Tsang A, Hausenloy DJ, Mocanu MM, Carr RD, Yellon DM. Preconditioning the Diabetic Heart: The Importance of Akt Phosphorylation. ***Diabetes*** 2005: 54(8); 2360-2364

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Hausenloy DJ, ***Tsang A***, Yellon DM. The Reperfusion Injury Risk Kinase (RISK)-pathway: A Common Target for Both Ischemic Preconditioning and Postconditioning. ***Trends in Cardiovasc Med*** 2005: 15(2); 69-75

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LIST OF ABBREVIATIONS

The following is a list of abbreviations used in this thesis

5-HD	5-hydroxydecanoic acid
8-SPT	8-(p-sulfophenyl) theophylline
A_1	adenosine type 1 receptor
A_{2a}	adenosine type A_{2a} receptor
AAR	area at risk
Akt	cellular Akt/protein kinase B
ANOVA	analysis of variance
ANT	adenine nucleotide translocase
APS	ammonium persulphate
ATP	adenosine triphosphate
ATPase	ATP synthase
AU	arbitrary units
BAD	Bcl-X_L/Bcl-2-associated death promoter
BAX	Bcl-associated X protein
BCA	bicinchoninic acid
BSA	bovine serum albumin
CAD	coronary artery disease
Ca^{2+}	calcium ion
CFR	coronary flow rate

CREATE-ECLA	Clinical Trial of Metabolic Modulation in Acute Myocardial Infarction Treatment Evaluation - Estudios Cardiologicos Latinoamerica
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemi-luminescence
eNOS	endothelial nitric oxide synthase
ERK (p42/44)	extracellular signal-regulated kinase
GIK	glucose insulin potassium
GK	Goto-Kakizaki
GLP-1	glucagon-like peptide 1
GLUT-4	glucose transporter type 4
GPCR	G-protein coupled receptor
G -protein	guanine nucleotide binding regulatory protein
GSK3β	glycogen synthase kinase 3β
H^+	hydrogen ion/proton
HbA1c	glycosylated (glycated) haemoglobin
HOE 642	Cariporide
HRP	horse radish peroxidase
iNOS	inducible nitric oxide synthase
IPC	ischaemic preconditioning
IRS-1	insulin receptor substrate-1
JNK	c-Jun NHP_2 terminal kinase
K^+	potassium ion

K_{ATP}	ATP-sensitive potassium channel
LAD	left anterior descending
L-NAME	N ω-nitro-L-arginine methyl ester
LY294002	2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride
MAPK	mitogen activated protein kinase
MEK	MAPK/ERK kinase
mito K_{ATP}	mitochondrial K_{ATP}
mM	micromolar
mPTP	mitochondrial permeability transition pore
Na^+	sodium ion
NADH	nicotinamide adenine dinucleotide
NIM811	(Melle- 4) cyclosporin
NO	nitric oxide
ODQ	1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one
p38 MAPK	38 kDa mitogen activated protein kinase
p70S6K	70 kDa ribosomal protein S6 kinase
PAGE	polyacrylamide gel electrophoresis
PCI	percutaneous coronary intervention
PD98059	2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one
PDK-1	3-phosphoinositide-dependent protein kinase 1
PI3K	phosphatidyl inositol 3-OH kinase
PIA	R-N6-(2-phenylisopropyl) adenosine
PKC	protein kinase C
PLSD	protected least significance difference

PMA	phorbol 12-myristate 13-acetate
PMSF	phenyl methyl sulphonyl fluoride
Postcon	postconditioning
RISK	reperfusion injury salvage kinase
ROS	reactive oxygen species
RPP	rate pressure product
SB 216763	3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H pyrrole-2,5-dione
SDS	sodium dodecylsulphate
SEM	standard error of the mean
SWOP	second window of protection
TEMED	N, N,N', N'-tetramethylenediamine
TGFβ	transforming growth factor β
TRIS	Trizma base
TTC	triphenyltetrazolium chloride
VDAC	voltage-dependent anion channel
ZM241385	2-(3)H]-4-(2-[7-amino-2-(2-furyl)(1,2,4)triazolo(2,3-a)(1,3,5,)triazin-5-yl amino]ethyl)phenol

Chapter One: GENERAL INTRODUCTION

1.1 Ischaemic Heart Disease

Ischaemic or coronary heart disease (CAD) is the leading cause of death in the Western world and represents one of the major burdens on healthcare systems today[1]. The spectrum of CAD ranges from "silent" or sub-clinical angina through to lethal myocardial infarction. For those patients with chronic angina and those fortunate to survive a myocardial infarction, the sequelae of CAD will continue to have a major impact on quality of life and to place an enormous strain on healthcare resources. Unless major advances are made in both the prevention and treatment of the consequences of CAD, its place as the primary cause of death in the Western world will continue for the foreseeable future. Targeting those strategies which limit the damage sustained as a result of a lethal ischaemic insult has been a major goal for both those involved in cardiovascular research and clinical medicine for many years.

Critical stenosis of a coronary artery occurs when the lumen of the artery is occluded by at least 70% and without adequate treatment, is usually the prelude to a complete coronary occlusion, an event which is ultimately lethal for the myocyte. The prerequisite for rescuing viable myocardium and reducing the mortality and morbidity as a consequence of an acute myocardial infarction is the early restitution of coronary flow i.e. reperfusion, after the ischaemic event by such means as thromobolytic drugs, primary coronary angioplasty, or coronary artery by-pass graft surgery. Lethal myocyte injury results as a

consequence of the physiological changes that occur from both ischaemia and reperfusion, as process known as ischaemic-reperfusion injury.

1.2 Ischaemic-Reperfusion Injury

The events following an acute coronary occlusion which lead to lethal myocyte injury are the result of several biochemical and metabolic perturbations that occur as a consequence of both the ischaemia and the restoration of coronary blood flow i.e. reperfusion. Cessation of coronary blood flow starves the myocardium of essential oxygen and metabolites and is inadequate to sustain oxidative metabolism due to the reduced electron flow along the electron transport chain. Several changes occur including depletion of important energy reserves such as adenosine 5'-triphosphate (ATP), which leads to increased anaerobic glycolysis. The build up of lactic acid as a result of anaerobic glycolysis leads to a reduction in intracellular pH and increase in intracellular acidosis[2]. In an effort to lower the concentration of hydrogen ions in the cytoplasm, the sodium/hydrogen ion (Na^+/H^+) exchange pump is switched on leading to the accumulation of cytosolic Na^+ in exchange for H^+. In order to combat the accumulation of the Na^+, the activation of the Na^+K^+-ATPase pump usually occurs, however this pump is inhibited in ischaemia due to the depletion of ATP. Activation of the Na^+/Ca^{2+} exchange pump in reverse mode, attempts to reduce the level of intracellular Na^+ but leads to the accumulation of intracellular and mitochondrial Ca^{2+} as a consequence. The rise in cytosolic Ca^{2+} leads to overstimulation of the myocardial actin-myosin contraction apparatus and myocardial hypercontracture ensues.

As already mentioned, the prerequisite for rescuing viable myocardium is the early restitution of coronary flow i.e. reperfusion, after the ischaemic event by such means as thromobolytic drugs, primary coronary angioplasty, or coronary by-pass graft surgery. However, reperfusion is not a completely benign process and can induce myocyte death, a phenomenon known as lethal reperfusion injury[3], which is now known to involve the processes of necrosis and apoptosis[4;5]. At the onset of reperfusion, the supply of oxygen and essential nutrients to the myocyte is restored. Reactive oxygen species (ROS) as a result of reoxygenation of the electron transport chain are generated, along with an increase in mitochondrial Ca^{2+} load. Mechanical changes which occur in the transition from ischaemia to reperfusion lead to pressure overload and resultant myofibrillar stretching. This ultimately leads to myocardial oedema, hypercontracture and myocyte death[6]. The damaging effects of both ischaemia and reperfusion on the myocyte is known as ischaemic-reperfusion injury.

The chronological events leading to lethal myocyte injury, provide two windows of opportunity for intervention using cardio-protective strategies to curtail the evolving myocardial infarction, namely the period **prior** to the onset of the lethal ischaemic insult which has its limitations in that the protective intervention must be applied prior to the index ischaemic event, which in the clinical setting of acute myocardial infarction is unpredictable and impractical, and the period **following** the lethal ischaemic insult which is more favourable since the onset of reperfusion is more predictable and is under the control of the operator. In this setting, novel cardio-protective strategies may therefore be applied as adjunctive therapy to current reperfusion strategies. Two such

strategies, ischaemic **pre**conditioning (IPC) and ischaemic **post**conditioning (Postcon) are the most potent cardio-protective phenomena known and will be discussed in more detail below.

1.3 Ischaemic Preconditioning

The concept of IPC arose from initial studies in 1986 from Reimer and colleagues[7] who demonstrated that four cycles of 10 minutes coronary artery occlusion followed by an intervening 20 minutes of reperfusion, did not result in increased myocardial necrosis or ATP depletion, compared with a single 10 minute occlusion or sustained ischaemia from a 40 minute coronary occlusion in an open chest dog model. Following these observations, the same group went on to describe a phenomenon in a study that has been heralded as a major breakthrough in the research of myocardial protection[8]. Using an in vivo canine model, they showed that 4 cycles of 5 minutes occlusion and 5 minutes reperfusion of the circumflex coronary artery applied prior to a sustained 40 minute occlusion, reduced myocardial infarct size by 75% compared to the control hearts. This phenomenon they described as "preconditioning with ischaemia" or as it is now known, ischaemic preconditioning. However, if the sustained ischaemic episode was extended to 3 hours, this effect was lost, suggesting that IPC merely serves to delay myocyte death or, that reperfusion of the myocardium is an essential component in the infarct reducing process. In other words, IPC renders the myocyte more tolerant to longer periods of ischaemia than would otherwise be expected. The preconditioning-induced reduction in infarct size was not a transient effect but a perdurable one, since

the investigators demonstrated the reduction in infarct size after 4 days of reperfusion. This strategy has since received an enormous amount of interest over the past two decades and is recognized as the most powerful endogenous cardioprotective mechanism known to man. IPC has been shown to be effective in all animal species studied so far, including mice[9], rats[10], rabbits[11], sheep[12], pigs[13], guinea pigs[14], and humans[15]. In addition, many pharmacological preconditioning mimetics have been described since[16]. Although the actual mechanisms by which IPC renders the myocardium more resistant to the effects of ischaemic-reperfusion injury are not fully understood, these studies and many hundreds since have provided us with invaluable information regarding cellular adaptation to stress, cell signalling and viability.

1.3.1 Chronology of Ischaemic Preconditioning

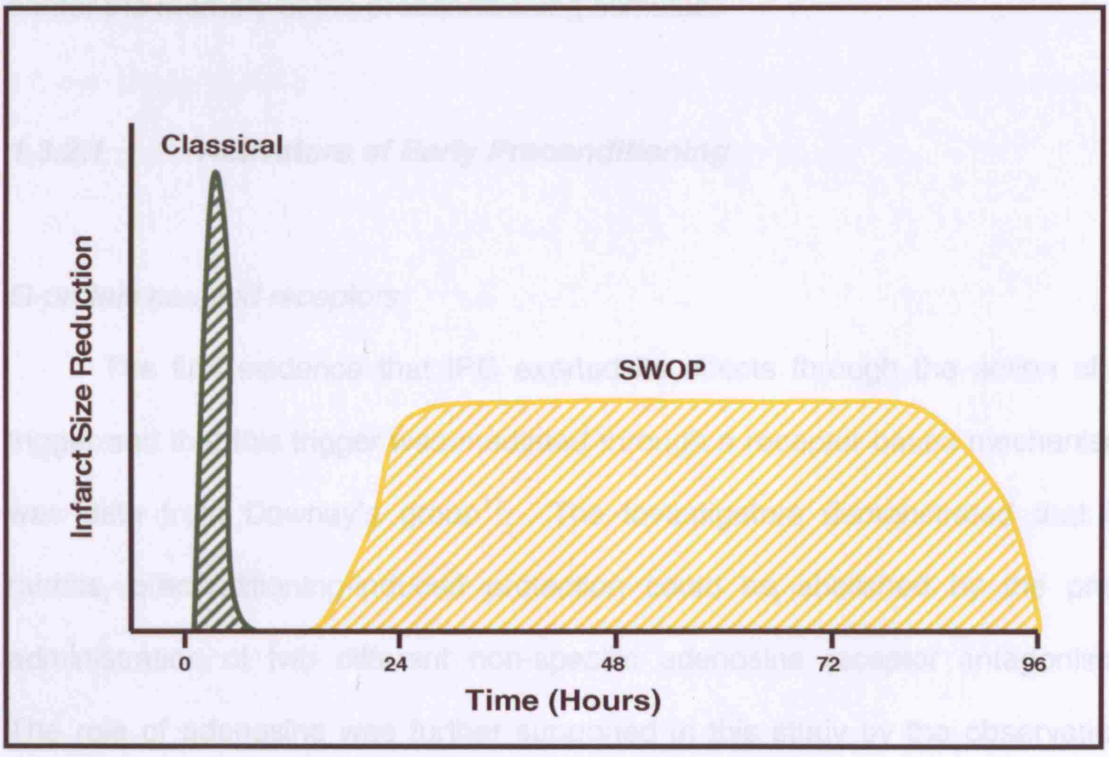
The beneficial effect of IPC is now known to have temporal characteristics which are biphasic in nature as shown in Figure 1.1.

Following the preconditioning stimulus, a 2-4 hour period exists within which the sustained ischaemia must occur in order for the myocardium to profit from the beneficial effects of infarct limitation after which the capacity for preconditioning disappears. This transitory form of IPC is referred to as *early or classical preconditioning.*

Within 24 hours of the preconditioning stimulus however, a further period of protection reoccurs that remains for up to 72 hours and has been termed the *second window of protection (SWOP).* Both of these forms of preconditioning have similar properties with regards to the triggers and signalling cascades that

mediate their protective effects however, early preconditioning appears to confer its protection through "immediate" end effectors rather than through the effects on protein translation and synthesis as in the SWOP. Whilst the end points with which to observe the outcomes from IPC include the effects on myocardial stunning, arrhythmias, recovery of contractile function as well as infarct size, the models used in this work focus on early preconditioning-induced reduction of infarct size and further reference to SWOP and other end points will not be made.

Figure 1.1: The Chronological Events in Ischaemic Preconditioning. Diagram showing the biphasic nature of preconditioning. The *early or classical* window appears immediately and lasts for 2-4 hours, whilst the *second window of protection (SWOP)* reappears within 24 hours although less potent, the SWOP has a longer duration. (Adapted from Yellon et al[17])



1.3.2 Mechanisms Involved in Early Preconditioning

A detailed description of all the cellular mechanisms that contribute to the phenomenon of IPC is beyond the scope of this thesis. However, a concise overview of those mechanisms which play key roles will follow. When visualizing mechanisms involved in preconditioning it is useful to consider those which act prior to the index ischaemia and those which act during or after the index ischaemia (or during reperfusion). The components whose effects are exerted prior to the index ischaemia should be regarded as triggers since these activate a myocyte adaptation induced by the preconditioning stimulus to resist infarction. The components whose effects are exerted during or after the onset of the index ischaemia should be considered as mediators or end effectors since these mechanisms "translate" the adaptation into infarct reduction. Linking the triggers to the end effectors are protective signalling pathways that confer the memory of the preconditioning stimulus.

1.3.2.1 Activators of Early Preconditioning

G-protein coupled receptors

The first evidence that IPC exerted its effects through the action of a trigger and that this trigger was mediated through a receptor based mechanism was data from Downey's group[11]. The investigators demonstrated that in rabbits, preconditioning-induced protection could be abolished by the prior administration of two different non-specific adenosine receptor antagonists. The role of adenosine was further supported in this study by the observation

that an intra-coronary infusion of adenosine could mimic the protection afforded by preconditioning. Similarly, the administration of an A_1 adenosine receptor agonist also conferred protection. Further support that preconditioning could be activated via ligand-induced G-protein coupled receptor (GPCR) mechanisms was provided by several studies implicating the role of opioids[18], bradykinin[19], acetylcholine[20], norepinephrine[21], and angiotensin[22]. Not all of these triggers are released by the myocardium during ischaemia and therefore are not all implicated in IPC however, they may still induce a preconditioned state by exogenous administration and occupation of their respectively expressed receptors. GPCR activated preconditioning does not rely solely on the population of a single subtype of GPRC, since the pharmacological blockade of one receptor subtype merely raises the threshold for protection when activated by other subtypes rather than completely abolishing it, as demonstrated by Goto and colleagues[19]. In this study, the investigators demonstrated that pharmacological inhibition of the bradykinin receptor abolished the protection from a single cycle of IPC, but not from multiple cycles indicating that several receptors may act in concert to reach the elevated threshold[19].

Reactive Oxygen species

The role of reactive oxygen species (ROS) as a trigger for IPC was implicated in studies which demonstrated that administration of a free radical donor could generate a preconditioned state[23;24], whilst pretreatment with a free radical scavenger could attenuate preconditioning's protective effect[25]. The actual mechanism by which ROS is thought to trigger IPC is not fully understood however, it is believed that ROS released from the inner

mitochondrial membrane[26] possibly through activation of the phosphatidyl inositol 3-OH kinase (PI3K)-Akt pathway[27], directly activates protective kinase pathways such as p42/44[28], protein kinase C (PKC)[23;29] and p38 MAP kinase[30], which mediate the effects of IPC.

K_{ATP} Channel

The distinction between triggers and mediators/end effectors is not clear cut as illustrated by the role of the K_{ATP} channel which has been postulated to possibly have dual roles as both a trigger and end effector. In this section the role of the K_{ATP} channel as a trigger will be discussed.

The K_{ATP} channel was first described in 1983 by Noma[31] and its role in ischaemic preconditioning has been established over the past decade or so, although somewhat controversial. Support for the role of K_{ATP} channels in preconditioning was first proposed by Gross and colleagues[32] who showed that the administration of the non-selective K_{ATP} blocker glibenclamide or 5-hydroxydecanoic acid (5-HD) either immediately before or after IPC, abolished the effect of infarct reduction. Two types of K_{ATP} channels are now known to populate the cardiomyocyte namely the sarcolemmal (surface K_{ATP}) and the mitochondrial (mito K_{ATP}) channels however, the majority of evidence suggests that it is the latter which plays an important role in preconditioning. Garlid's group were the first to implicate the mito K_{ATP} channel in cardio-protection by initially demonstrating that mito K_{ATP} channel opening was 2000 times more sensitive to diazoxide in addition to showing that 5-HD inhibited mito K_{ATP} channels rather than sarcolemmal K_{ATP} channels[33]. Subsequently, the same group demonstrated that at micromolar concentrations, diazoxide could protect

isolated rat hearts from infarction without any effect on sarcolemmal K_{ATP} channels[34]. Evidence for a triggering role was demonstrated in both isolated rat[35] and rabbit[36] hearts in which a 5 minute administration of the mito K_{ATP} opener diazoxide, followed by a washout, protected the hearts from a subsequent prolonged ischaemic insult. In these experiments, it would have been expected that the mito K_{ATP} channel would have been closed prior to the index ischaemia however, protection from infarction was observed suggesting that the transient opening of the mito K_{ATP} channel by diazoxide, had triggered a preconditioned state. Furthermore, when Pain and colleagues studied the effect of timing of this mito K_{ATP} opening by the administration of the mito K_{ATP} channel blocker 5-HD, they found that protection was only blocked when 5-HD was given during the preconditioning protocol and not during the index ischaemia[36]. Taken together, these observations suggested that opening of the mito K_{ATP} channel acts as a trigger of preconditioning. Supportive data again using 5-HD to inhibit mito K_{ATP} channel opening in rabbits during the preconditioning stimulus or during the index ischaemia came from Wang et al[37] however, this group also showed that provided the concentration of 5-HD was increased fourfold they could also abolish protection when administered during the index ischaemia which suggests a mediator role. Although these authors amongst others[38] postulated that mito K_{ATP} channels may have a dual role as both triggers and mediators, the fact that the fourfold increase in 5-HD concentration may have had a non-specific effect cannot be excluded. Despite the evidence that the mito K_{ATP} channel may act as a trigger in IPC, where the mito K_{ATP} channel fits into the signal transduction pathway is not fully understood. Data that suggests that the mito K_{ATP} channel activates

downstream kinases comes from Downey's group[36] who were able to inhibit the protection afforded by diazoxide-induced mito K_{ATP} channel opening in rabbit hearts by the use of the tyrosine kinase blocker genistein, but not by the administration of the protein kinase C (PKC) blocker chelerythrine, whilst data from Wang et al[35], demonstrated inhibition of diazoxide's protection by the administration of chelerythrine in rat hearts. These studies imply that the mito K_{ATP} channel is located upstream and acts as a trigger in IPC by the activation of downstream kinase pathways.

The answer to how mito K_{ATP} channel opening actually triggered the activation of kinases was first provided by Murphy's group[39] who demonstrated that the free radical scavenger *N*-acetylcysteine, could block the diazoxide-induced protection in isolated rat hearts using functional recovery as an end point. In addition, the same group showed that diazoxide resulted in free radical generation in isolated cardiomyocytes which could be inhibited by 5-HD. The importance of the role of free radicals was subsequently supported by studies from other groups[36;40-43]

1.3.2.2 *Signal Transduction in Early Preconditioning*

The Phosphatidylinositol 3-OH Kinase (PI3K)-Akt Pathway

The contribution of the phosphatidylinositol 3-OH kinase (PI3K)-Akt signalling pathway in IPC was first implicated by Tong et al[44] who demonstrated that in isolated perfused rat hearts using functional recovery as an end point, IPC induced the phosphorylation of Akt prior to the index ischaemia and importantly that this effect could be abolished by the pharmacological inhibition

of PI3K using Wortmannin. Subsequent work from the same group showed that IPC-induced phosphorylation of the PI3K-Akt cascade led to the phosphorylation and inactivation of a downstream target glycogen synthase kinase 3β (GSK3β), an effect which was blocked by Wortmannin. Prior to the index ischaemia, administration of two separate GSK3β inhibitors, lithium and SB 216763 mimicked the protective effects of IPC[45]. The importance of the PI3K-Akt pathway in IPC has been confirmed by further studies from Yellon's laboratory[46].

Where the PI3K-Akt cascade is located with respect to preconditioning is not fully clear but has been suggested to convey the protective signal between the GPCR[47] via nitric oxide[48] and the mitochondrial release of ROS[27;42] by data from Downey's group.

Protein Kinase C

In 1994 two studies, one from Downey's group[49] in rabbits and one from Yellon's group[50] in rats, were the first to implicate the role of protein kinase C (PKC) in IPC by the demonstration that specific PKC agonists induced a preconditioned state, whilst conversely inhibition of PKC abrogated preconditioning's protective effect. The location of PKC in the cascade of IPC was proposed by further work from the same group who demonstrated that IPC-induced protection in rabbit hearts could only be abolished if the PKC inhibitor staurosporine, was present during the index ischaemia rather than during the preconditioning protocol[51], suggesting PKC's role in IPC as a mediator and not as a trigger. As discussed earlier, the link between triggers of IPC acting prior to the index ischaemia and mediators acting during or after the index ischaemia

resides in the memory effect. The translocation of PKC from the cytosol to membrane results in its activation, and it was this relatively slow translocation of PKC that Downey's laboratory suggested was responsible for IPC's memory. Further evidence in support of PKC's location downstream to GPCR's came from Iliodromitis et al[52] who demonstrated that the combination of the adenosine receptor blocker 8-(p-sulfophenyl) theophylline (SPT) and one of two PKC agonists, either phorbol 12-myristate 13-acetate (PMA) or phenylephrine protected rabbit hearts from ischaemic-reperfusion injury. Conversely, the combination of the adenosine receptor agonist R-N6-(2-phenylisopropyl) adenosine (PIA) and the PKC blocker chelerythrine had no protective effect[52]. Taken together, these findings suggest that PKC is located downstream to adenosine receptors in IPC.

Exactly how PKC mediates the effects of IPC-induced cardio-protection, whether via additional signalling pathways or end effectors, is not clear.

Tyrosine Kinase

Tyrosine kinase's role in IPC was first proposed in a study by Maulik and colleagues[53] using the tyrosine kinase inhibitor genistein to block IPC protection in isolated rat hearts. The evidence that placed tyrosine kinase downstream of PKC came from a study by Baines et al[54]. Using rabbit hearts the investigators demonstrated that the tyrosine kinase blockers genistein and lavendustin-A could only abolish protection from IPC if they were administered at the onset of the index 30 minute ischaemia and not if given to bracket the IPC stimulus. Furthermore, PKC activation by phorbol 12-myristate 13-acetate (PMA) resulted in protection comparable with IPC which was also sensitive to both genistein

and lavendustin-A. Conversely, although the action of anisomycin, a tyrosine kinase agonist was protective, the presence of chelerythrine did not affect anisomycin-induced protection[54]. From these observations, the authors concluded that PKC was upstream of tyrosine kinase in the signal transduction cascade. The actions of tyrosine kinase on mitogen-activated protein kinases may be a possible explanation for its involvement in myocardial protection.

Mitogen-Activated Protein Kinases

The class of mitogen-activated protein kinases (MAPK) can be divided into further subclasses namely, p42/44 (42 and 44 kDa) extracellular signal regulated kinase (ERK1/2), p38 (38 kDa) MAPK, and the *c-jun* (46 and 54 kDa) kinase (JNK). These MAP kinases are activated by dual phosphorylation by tyrosine kinase (also known as MAPK kinase).

Although some studies have demonstrated that IPC results in phosphorylation of p42/44 (ERK1/2) [46;55], only a few have demonstrated that IPC-induced protection was actually dependent on this phosphorylation[56;57], and therefore a causal role for ERK1/2 as a necessary signal transduction pathway in IPC remains unresolved.

Data suggesting the role of p38 MAPK in IPC is inconsistent. Whilst several different isoforms of p38 MAPK has been described, the α-isoform has been implicated to mediate cardiomyocyte apoptosis, in contrast to the β-isoform which is anti-apoptotic[58]. More complicated is the involvement of p38 MAPK in IPC-induced cardioprotection. In this regard, some groups have demonstrated that p38 MAPK is increased during the index ischaemia in preconditioned rabbit[59;60] and rat[53] hearts suggesting a protective effect, whilst

others have reported that p38 MAPK is inconsistent in preconditioned pig hearts[61]. In contrast, decreased p38 MAPK levels have been reported during the index ischaemia in preconditioned rat[62] and rabbit[63] models suggesting a detrimental effect of p38 MAPK activation.

In a similar manner, the role of JNK MAPK in IPC is inconsistent. While both JNK 46 and 54 have been shown to be present in the myocardium[64], some studies have demonstrated that activation of JNK occurs in the setting of ischaemia-reperfusion[65;66], and that this activation can be completely abolished by the PKC inhibitor chelerythrine, indicating that PKC is upstream to and mediates JNK activation at least in rabbits[67]. In contrast, other studies have shown that JNK protein levels are no different when compared with control hearts during the index ischaemia[60].

1.3.2.3 *End Effectors of Early Preconditioning*

The actual mechanism or end effector which renders the myocyte resistant to a subsequent lethal ischaemic insult has been a subject of debate for several years. Several important components have been proposed to be the end effector however, two of these namely the mito K_{ATP} channel and the mitochondrial permeability transition pore (mPTP) have emerged as the most likely candidates.

Sodium/Hydrogen Ion Exchanger

As discussed earlier in section 1.2, the activation of the Na^+/H^+ exchange pump as a result of ischaemic injury is deleterious, in that it results in an

increase in intracellular Na^+ and consequently through the reverse mode action of the Na^+/Ca^{2+} exchanger, an accumulation of intracellular and mitochondrial Ca^{2+} load. Therefore, inhibition of this exchanger would seem an obvious target by which to limit lethal myocyte injury and has proved to be a very powerful strategy in myocardial protection to date[68]. In this regard, Xaio and colleagues[69] using the Na^+/H^+ exchanger blocker HOE 642, found that in isolated perfused rat hearts, the activity of the Na^+/H^+ exchanger was reduced in preconditioned hearts in early reperfusion compared with controls as measured by intracellular Na^+ concentrations. Administration of HOE 642 at the beginning of reperfusion significantly improved the contractile recovery of ischaemic hearts, an effect comparable to IPC. However, the effect of HOE 642-induced protection and IPC were not additive when combined. Further support for Na^+/H^+ exchanger inhibition came from work from Downey's group[70] who demonstrated that Na^+/H^+ pump inhibition with the blocker amiloride, protected rabbit hearts to a similar extent as IPC. Furthermore, this group were unable to abolish the protection afforded by amiloride using either the PKC inhibitor polymyxin B, or the K_{ATP} channel blocker glibenclamide[70]. These observations support the notion that the location of the Na^+/H^+ exchanger is downstream to the K_{ATP} channel and mediator PKC, strengthening its role as an end effector. This hypothesis unfortunately, is not straightforward in that PKC mediated protection in IPC has been shown to be as a consequence of activation of the Na^+/H^+ exchanger, rather than its inhibition. The protective effects of Na^+/H^+ exchanger blockade may be due to the effects on reducing intracellular Ca^{2+} levels. However, what remains to be clarified is that since water accompanies the accumulation of intracellular Na^+ as a result of

ischaemic-reperfusion injury, the beneficial effects on the myocyte may be the consequence of osmotic swelling reduction.

Apoptotic Effects

The contribution of apoptosis or programmed cell death to the extent of infarction as a result of an ischaemic-reperfusion insult is unclear, but has been proposed by Engler's group[71] to play an important role. Others have implicated that IPC may reduce apoptotic cell death by reducing the level of the pro-apoptotic protein BAX[72] and attenuation of caspase activation and DNA fragmentation[73].

Reactive Oxygen Species

The production of free radicals at the onset of reperfusion of the ischaemic myocardium has been suggested to be a major contribution to lethal reperfusion injury[74]. In a similar manner to other end effectors, the evidence is conflicting as demonstrated by the fact that the level of free radical scavengers has been found to be higher in preconditioned rat hearts[75] whilst conversely, in rabbit hearts no demonstrable differences were observed between preconditioned and control hearts[76]. In support of the free radical end effector, Vanden Hoek and colleagues observed that hypoxic or adenosine-induced preconditioning led to attenuation of the oxidant burst seen at the onset of reperfusion in chick cardiomyocytes[77], and furthermore, this preconditioning-induced attenuation of oxidant stress was dependent on PKC activation and K_{ATP} channel opening.

Gap Junctions

Connexin 43, the major component of myocardial gap junctions allows the relay of signals between adjacent myocytes. It is this relay of signals which may be responsible for permitting the spread from cell to cell of those factors leading to myocyte death in ischaemia-reperfusion[78]. The importance of connexion 43 was observed in a study in which protection against infarction by IPC was lost in transgenic mice deficient in connexion 43. Whether it is the closing or opening of the gap junction which mediates the protective effect of IPC is unclear at this time. In support of the latter, Przyklenk's laboratory blocked the effects of IPC in isolated mouse hearts using the gap junction blocker heptanol[79]. In contrast, data from Garcia-Dorado et al[80] in 1997 demonstrated that heptanol afforded protection in cardiomyocytes, isolated rat hearts and in vivo pigs. Later, these findings were confirmed in a study using rabbit hearts by Saltman and colleagues[81] and in addition, they demonstrated that the protective effects of heptanol were not prevented by pretreatment with glibenclamide. These observations support the notion that it is the closure of gap junctions that enables protection and that gap junction-induced protection is not mediated via a mechanism involving the K_{ATP} channel.

The Mitochondrial K_{ATP} Channel

Although there is convincing evidence that the mito K_{ATP} channel can act as a trigger in IPC, there is also data to support its role as an end effector, with many authors now proposing a dual role. One plausible theory would be that the triggering mechanism of the mito K_{ATP} channel leads to the generation of ROS which activates signal transduction pathways during the IPC stimulus.

These kinases and their downstream targets feedback to the mito K_{ATP} channel during or after the index ischaemic episode to stimulate its end effector role. In this regard, studies have demonstrated that inhibition of the mito K_{ATP} channel at reperfusion with glibenclamide or 5-HD blocks the protective effects of IPC[32;82], and on a similar note, the opening of the mito K_{ATP} channel using the potent opener diazoxide, led to cardioprotection[34;83]. How mito K_{ATP} channel opening actually leads to cardio-protection in unknown but has been suggested that it is the prevention of mitochondrial calcium overload, which as discussed earlier is detrimental to the myocyte, is the mechanism. Indeed, support for this has been implicated by Terzic's laboratory who found that the K_{ATP} openers, diazoxide and pinacidil prevented calcium overload in isolated cardiac mitochondria. The mechanism for reduced mitochondrial overload is presumed to be due to depolarization of the mitochondrial membrane which reduces the electrochemical gradient for calcium entry however, in ischaemia the mitochondrial membrane is reduced and the propensity for depolarization by mito K_{ATP} channel opening is greatly diminished. In this respect, research has focussed on another potential candidate as a possible end effector.

The Mitochondrial Permeability Transition Pore

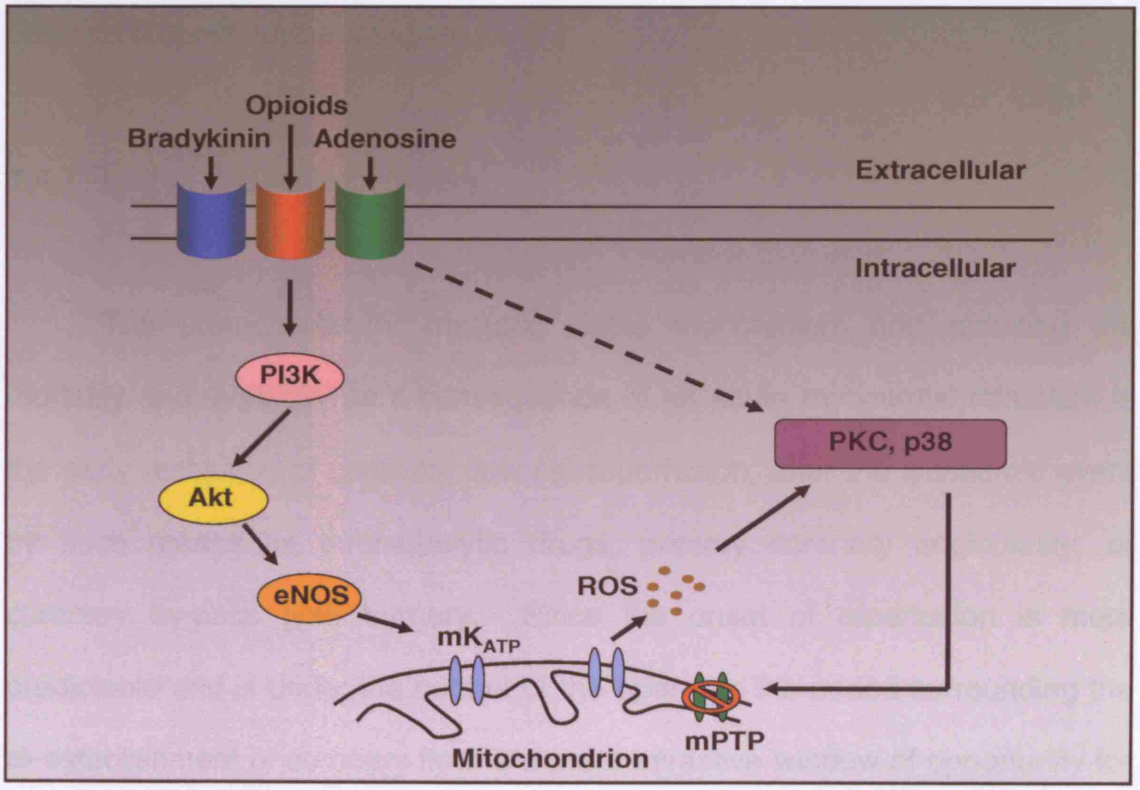
The mPTP formation occurs as a result of the association between adenine nucleotide translocase (ANT) in the inner mitochondrial membrane, the voltage-dependent anion channel (VDAC) and mitochondrial cyclophilin, and opening of this pore has been postulated to occur as a consequence of events which occur at reperfusion[84]. These include an influx of calcium into the mitochondrial matrix due to depolarization of the mitochondrial membrane, a

return to pre-ischaemic pH levels with the onset of myocardial reperfusion, and exposure to a free radical burst due to reoxygenation of the electron transport chain. The mPTP then acts as a gateway into the mitochondria and opening of this pore permits the influx of injurious substances into the mitochondria causing swelling, damage and ultimately myocyte death.

Novel data linking the protective effect of IPC with the mPTP came from Hausenloy et al[85] who found that isolated rat hearts could be protected from infarction by IPC, cyclosporin A (an inhibitor of mPTP opening)[86], and pre-treatment with diazoxide or an adenosine A_1 receptor agonist. This observed protection could be abolished in the presence of atractyloside, an mPTP opener at reperfusion providing compelling evidence that inhibition of mPTP opening at reperfusion may be the end effector of early preconditioning.

Combining the evidence so far, the interplay between GPCR activation, triggers, signal transduction pathways and mito K_{ATP} channel opening in early preconditioning, is proposed in Figure 1.2.

Figure 1.2: The Interplay Between GPCR Activation, Signal Transduction and the Mitochondrial K_{ATP} Channel in Early Preconditioning. Hypothetical scheme representing the interaction between components of early preconditioning to induce myocardial protection. Activation of G-protein coupled receptors (GPCR) by bradykinin or opioids via the phosphotidylinositol 3-OH kinase (PI3K)-Akt pathway, results in mitochondrial reactive oxygen species (ROS) release through mitochondrial K_{ATP} channel opening. ROS then activates downstream kinases such as protein kinase C (PKC) to act on the end effector and possibly prevent mitochondrial permeability transition pore (mPTP) opening. Activation of PKC may occur directly by the triggering of adenosine receptors. (Adapted from Yellon et al[17])



1.4 Targetting the Reperfusion Period

Despite IPC being one of the most powerful endogenous cardio-protective mechanisms known to man, this phenomenon has its limitations in that it requires prior knowledge of the onset of the index ischaemia in order to apply the preconditioning strategy prior to the ischaemic event, which in the clinical setting of acute myocardial infarction is unpredictable and impractical. Although preconditioning has given us invaluable information regarding cellular adaptation to stress, cell signalling and viability, it in itself, has failed to find a clinical niche. However, based upon our understanding of the mechanisms associated with preconditioning, this has allowed us to revisit and focus on other cardioprotective strategies.

1.4.1 Lethal Reperfusion Injury

The prerequisite for rescuing viable myocardium and reducing the mortality and morbidity as a consequence of an acute myocardial infarction is the early restitution of coronary flow i.e. reperfusion, after the ischaemic event by such means as thromobolytic drugs, primary coronary angioplasty, or coronary by-pass graft surgery. Since the onset of reperfusion is more predictable and is under the control of the operator, the period surrounding the re-establishment of coronary flow is a more attractive window of opportunity for protection from the consequences of both the ischaemia and reperfusion-induced injury[87]. Novel cardio-protective strategies, which protect the myocardium from the detrimental effects of ischaemia-reperfusion injury, may

therefore be applied as adjunctive therapy to current reperfusion strategies. However, reperfusion is not a completely benign process and can induce myocyte death, a phenomenon known as lethal reperfusion injury[3], which is now known to involve the processes of necrosis and apoptosis[71;88]. The concept of reperfusion-induced injury is controversial, being initially supported by most groups but then falling out of vogue[89]. More recently however, based on new hypotheses and a better understanding of cellular signalling, it seems that the concept has now returned full circle with the advent of pharmacological agents that have been shown to reduce myocardial cell death when given during the initial stages of reperfusion[90].

One such novel strategy targeting the reperfusion phase is the phenomenon known as ischaemic postconditioning (Postcon) which was first introduced in 2002 by Vinten-Johansen's group[91]. This phenomenon, in which the application of transient brief interruptions to reperfusion by ischaemic episodes, results in reduced myocardial injury, has lead to renewed interest in the development of protective manoeuvres to combat the effects of lethal reperfusion injury. However, ischaemic postconditioning as discussed below is likely to represent a form of modified reperfusion which paradoxically has been known for a number of years to be beneficial to the ischaemic myocardium.

1.4.2 Modified Reperfusion

The damaging effects of reperfusion are due to the many biochemical and physical perturbations that occur in the transition from ischaemia to reperfusion. Immediate full flow reperfusion leads to pressure overload and resultant myofibrillar stretching. This ultimately leads to myocardial oedema,

hypercontracture and myocyte death[87]. Other detrimental events are exposure to a sudden burst of free radicals, mitochondrial calcium overload, and increased endothelial dysfunction[92].

The beneficial effects of alterations to the conditions of reperfusion have been known since the mid 1980's. Studies from Buckberg and colleagues[93] in the dog model reported that contractile recovery was improved in those animals that were subjected to a gradual relief of the left anterior descending (LAD) artery occlusion when compared to those with sudden relief. Work from Hori et al[94] a few years later described the importance of pH during the early stages of reperfusion. They found that maintaining a more acidic pH by staged reperfusion led to improved myocardial contractility and that the administration of an alkaline solution during this manouvere abolished the improvement in recovery. Other studies have demonstrated that gradual reperfusion leads to the accumulation of those metabolites necessary to achieve improved post-ischaemic functional recovery, namely ATP, glutamate, aspartate, and adenine nucleotides[95]. Data that followed from Vinten-Johansen's laboratory[96] reported that gradual reperfusion not only reduced endothelial dysfunction but also reduced infarct size in dogs subjected to left coronary artery ligation.

However, the concept of modified reperfusion as a cardioprotective intervention failed to make an impact. One potential reason for this may have been due to the controversy surrounding the very existence of lethal reperfusion injury, with some groups questioning the existence of reperfusion injury as a distinct entity, believing instead that reperfusion simply augmented the damaging effects of the ischaemic insult[97]. Others believed that the metabolic changes of reperfusion itself could induce cellular injury without an ischaemic

period[98;99]. More recently however, it has been shown that a range of pharmacological agents given at the moment of reperfusion following an ischaemic insult, can significantly protect the myocardium, and this effect has been shown to involve protection against necrosis and apoptosis. Furthermore, during the early stages of reperfusion there is an up-regulation of pro-survival kinases (termed the Reperfusion Injury Salvage Kinase - RISK pathway) which, taken together, has promoted a renewed interest in cardioprotective reperfusion strategies[100].

1.4.3 The Reperfusion Injury Salvage Kinase (RISK) Pathway

Whilst the mechanisms involved in reperfusion injury are known to involve apoptosis and necrosis amongst others, it is also realized that cells have an inherent program for survival following ischaemic-reperfusion insults, via the recruitment of innate pro-survival kinase cascades. The phosphatidylinositol-3-OH kinase (PI3K)-Akt, and MEK 1/2-p42/44 kinases have been shown to be important components of these cell-survival pathways[101] and have anti-apoptotic effects. Reduction in reperfusion-induced injury can be obtained by the up-regulation of these kinases[100]. In this regard, work from our group and others have shown that this can be achieved using pharmacological agents such as insulin[102], insulin-like growth factor 1[103], atorvastatin[104], bradykinin[105], urocortin[106], cardiotrophin 1[107], transforming growth factor-β1[108], and opioids[109]. Therefore, intervening at the time of reperfusion to attenuate the effects of lethal reperfusion injury provides an important strategy for cardio-protection. In this regard, the recently described phenomenon of postconditioning offers another

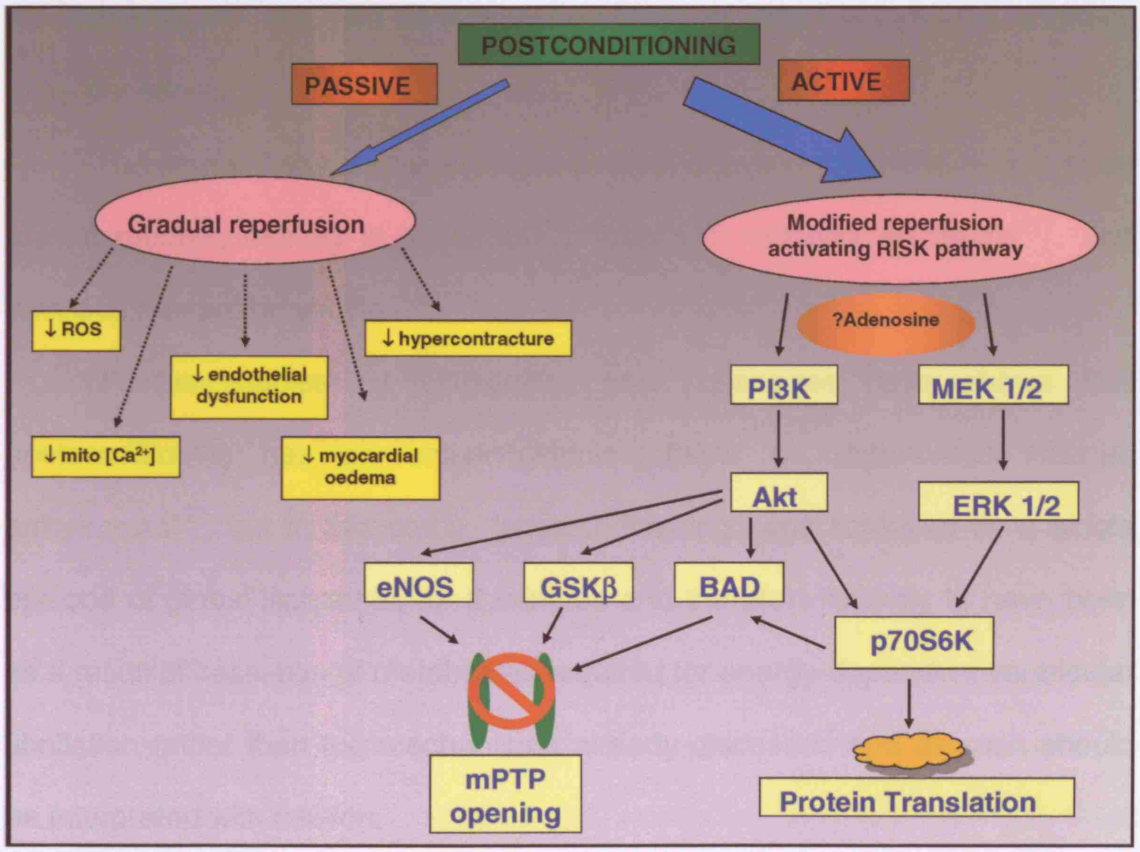
potentially effective intervention for limiting myocardial injury that appears to have many similarities to that described above.

1.4.4 Ischaemic Postconditioning

In 2003, a phenomenon termed ischaemic postconditioning (Postcon), a cardioprotective manoeuvre which targets the reperfusion phase, was first published by Vinten-Johansen's group[110]. Their study described the application of several brief, transient cycles of alternating reperfusion/ischaemia immediately following the sustained ischaemic episode, which resulted in a reduction in myocardial injury. In the in vivo dog model, at the immediate onset of reperfusion following a 60 minute occlusion of the LAD artery, postconditioning was achieved by allowing reflow for 30 seconds followed by 30 seconds reocclusion of the LAD artery repeated a total of 3 times prior to the remaining reperfusion phase[110]. Postconditioning reduced infarct size by 44%, an effect comparable with ischaemic preconditioning. However, in this study, the authors suggested that the mechanisms by which preconditioning and postconditioning conferred myocardial protection were likely to be different since preconditioning triggers protective pathways before ischaemia, whereas postconditioning alters events after ischaemia. Subsequent data from Yellon's group suggests that this may not be the case[111]. They demonstrated that by inhibiting PI3K using LY294002 or MEK1/2 using PD98059 during the first 15 minutes of reperfusion, the cardioprotective effects of ischaemic preconditioning could be abrogated. Taken together with data from this thesis as we shall see[112] and a recent study from Downey's group[113], postconditioning has been

demonstrated to activate the cellular pro-survival PI3K-Akt and MEK 1/2-ERK pathways and that by inhibiting these pathways, the protective effects of postconditioning are abolished. These studies suggest that both preconditioning and postconditioning may share a common pathway (see Figure 1.3).

Figure 1.3: The Cellular Signalling Pathways Involved in Ischaemic Postconditioning. Hypothetical scheme showing the possible mechanisms of protection induced by ischaemic postconditioning: 1) gradual reperfusion may have a 'passive' effect modifying reperfusion injury by a reduction in reactive oxygen species (ROS), mitochondrial calcium load, endothelial dysfunction, myocardial edema, and hypercontracture 2) upregulation of the Reperfusion Injury Salvage Kinase (RISK) pathway, an 'active' effect via activation of PI3K-Akt or ERK 1/2, phosphorylates downstream targets such as GSKβ, BAD/Bax, and eNOS producing nitric oxide which inhibits mPTP opening. Phosphorylation of p70s6K confers protection by inactivating BAD or through protein translation.



The term postconditioning refers to the ischaemic stimulus being applied after the lethal ischaemic period rather than prior to it as in preconditioning. This use of ischaemia after a prolonged ischaemic period is a simple and logical progression. The fact that a) brief ischaemia prior to prolonged ischaemia is protective, b) many pharmacological agents have since been found to be preconditioning mimetics, and c) many pharmacological agents have been shown to be protective when administered at early reperfusion, would lead one to speculate that brief ischaemia following a lethal episode may protect the myocardium from injury if postconditioning is mediated by the same cellular signalling pathways as drugs given at reperfusion. However, this should not detract from the fact that intermittent interruptions to reperfusion is physically modifying reperfusion, and as discussed earlier, modified reperfusion is known to be beneficial.

Subsequent studies have shown postconditioning to be effective in other animal models, namely in vivo rats[114], rabbits[113], isolated rat hearts[112], and neonatal rat cardiomyocytes[115;116].

Further studies by Galagudza and colleagues have shown that postconditioning has an anti-arrhythmic effects on reperfusion induced arrhythmias[117], but in this study, "postconditioning" was achieved by a single episode of global ischaemia for 2 minutes and therefore is likely to have been as a result of cessation of metabolites required for energy-dependent ventricular fibrillation rather than the mechanisms already discussed and as such should be interpreted with caution.

Mechanistic data was provided by subsequent work from Kin et al[114] who demonstrated that postconditioning was effective in open chest rats. In

addition, postconditioned hearts showed less free radical generation and less oxidant-induced injury as demonstrated by malondialdehyde levels and dihydroethidium fluorescence for superoxide generation. The role of oxidant injury at reperfusion was further supported by data from Sun et al[115] in rat cardiomyocytes exposed to hypoxic postconditioning. However, further studies are required to elucidate the source and exact role of free radicals which are reduced by postconditioning. Several interesting points were evident from Kin's data. Firstly, postconditioning was achieved using 3 or 6 cycles of 10 second interruptions to reperfusion compared with 30 seconds in the dog, suggesting that the duration of ischaemia of the postconditioning protocol may be species dependent. This fact is further supported by work from our laboratory which showed that postconditioning using 6 cycles of 30 second interruptions to reperfusion in isolated rat hearts failed to reduce infarct size (unpublished data). Furthermore, from Kin's data, the limitation of infarct size from 3 or 6 cycles of postconditioning were not statistically different from one another, suggesting that it is not the quantity of cycles that is important but the duration. In this regard, Schwartz et al[118] failed to demonstrate any infarct reduction in open chest pigs using a postconditioning protocol of 3 cycles of 30 seconds reperfusion/re-occlusion, the possible explanations being that in the pig species, the duration of interruption to reperfusion required is different to the dog in addition to the differences in coronary collateral flow that exist between these two species.

As discussed earlier, since several pharmacological agents have been shown to limit myocardial injury when administered at reperfusion by the activation of the pro-survival cellular kinase pathways PI3K-Akt and MEK1/2-

ERK, one may speculate that postconditioning exerts its protective effects by the same mechanisms[100].

1.4.4.1 Upstream Mediators of Postconditioning

Whilst the involvement of the RISK pathway has been demonstrated in postconditioning[112;113], how the RISK pathway is up-regulated is unclear. Adenosine has been implicated as a potential upstream mediator by two recent studies. The first from Vinten-Johansen's lab, using a mouse and rat model, demonstrated that the levels of endogenous adenosine were reduced in the coronary effluent collected shortly after completion of the postconditioning protocol, suggesting that greater intravascular adenosine was being retained as a result of postconditioning[119]. Furthermore, postconditioning's infarct sparing effect was abolished by the presence of both 8-SPT (a non-selective adenosine receptor antagonist) and ZM241385 (an A_{2a} receptor antagonist). The second study from Downey's lab also supported the inhibitory action of 8-SPT on postconditioning in rabbits[120]. Taken together, these suggest that adenosine may have an important role in the activation of the RISK pathway. Other mechanisms by which postconditioning activates the RISK pathway need to be further investigated for example, the possible role of PKC or ROS as upstream mediators.

1.4.4.2 Downstream Mediators of Postconditioning

Nitric oxide and mito K_{ATP} channels have been shown to be downstream targets in postconditioning from Downey's group[113]. Postconditioning has in addition, been shown to be blocked by L-NAME (a nitric oxide synthase inhibitor) and ODQ (a guanylyl cyclase antagonist) in both rabbits[120] and rats[121], implicating these as downstream components.

1.4.4.3 End-Effector of Postconditioning

A number of studies have discovered a common finding with regard to the timing of postconditioning. The protection induced can only be taken advantage of if postconditioning is initiated at the onset of reperfusion and is lost if delayed by 1 minute in the rat[114] and rabbit models[122]. Therefore, this would suggest that the end effector of protection must exert its actions during the initial stages of reperfusion. In this regard, pharmacologically inhibiting the mitochondrial permeability transition pore (mPTP) during the first few minutes of reperfusion[85;123] has been shown to be cardioprotective and delaying this inhibition by 15 minutes abolishes this protection. Similarly, delaying the administration of insulin, which is known to activate the RISK pathway, until after the first 15 minutes of reperfusion abrogates its infarct limiting effect[102]. Therefore, one could speculate that the mPTP, which is known to regulate cell death during the first few minutes of reperfusion[124], is potentially the main candidate as the end effector.

The importance of mitochondria in postconditioning was further supported in a study using a rat model of hepatic ischaemia-reperfusion injury which demonstrated that postconditioning of the liver resulted in a decrease in mitochondrial ultrastructural injury and apoptosis[125].

Evidence for mPTP involvement in postconditioning-induced protection has been strengthened by the important study from Ovize's lab[126]. Using isolated rat mitochondria, this group found that postconditioning reduced mitochondrial susceptibility to calcium overload compared to controls, an effect comparable with preconditioning and NIM811 (a cyclosporin analogue specific mPTP inhibitor). However, the mechanism by which postconditioning inhibits mPTP opening is still unclear, although subsequent work from the same lab suggests that this mPTP opening is PI3K regulated[127], as evidenced by the abrogation of mitochondrial resistance to calcium overload in the presence of Wortmannin or LY294002.

1.4.5 Clinical Implications of Postconditioning

Whilst the phenomenon of postconditioning is a novel and attractive strategy scientifically, in the clinical situation postconditioning may be difficult conceptually to introduce i.e. re-introduction of ischaemia at the time of reperfusion may lead to potential complications. For example, during primary angioplasty, repetitive inflations and deflations of the balloon may result in coronary plaque rupture with the consequences of re-stenosis or embolic events. Despite this however, early evidence is emerging that postconditioning applied at the time of angioplasty can indeed protect the human myocardium

from injury[128]. During coronary bypass surgery, interruptions to reperfusion via the newly grafted conduit may only lead to regional myocardial protection in the area supplied by that bypass. An alternative strategy during bypass surgery would be the repetitive clamping and unclamping of the ascending aorta to achieve ischaemic postconditioning, a concept however that many cardiac surgeons would be unwilling to perform due to the high risk of disrupting atheromatous plaque debris and subsequent risk of stroke. The re-introduction of ischaemia at the time of administration of thrombolytic drugs is also not feasible clinically.

However, the concept of 'pharmacological postconditioning' by the administration of agents which activate the RISK pathway and mediate the protective effects of postconditioning is a more practical solution. Agents such as insulin[102], atorvastatin[104], bradykinin[105], TGFβ[108], and GLP-1[129] could individually be used as adjuvants to current reperfusion strategies such as thrombolytics and percutaneous coronary intervention (PCI), to limit lethal reperfusion injury and could form the basis of much needed and important reperfusion strategies.

Furthermore, another clinically attractive phenomenon is the concept of remote postconditioning. As in remote preconditioning, there is some evidence that the application of postconditioning to an organ distant to the heart can lead to a reduction in myocardial injury from subsequent ischaemia-reperfusion[130].

1.5 Summary

Ischaemic preconditioning and postconditioning are the most powerful endogenous cardio-protective mechanisms known in the field of myocardial protection. IPC has its limitations in that it requires prediction of the onset of the lethal ischaemia in order to apply the preconditioning strategy, which in the clinical setting of acute myocardial infarction is near impossible and impractical. Studies of IPC over the past two decades have given us invaluable information regarding cellular adaptation to stress, cell signalling and viability, although in itself, has failed to find a clinical niche. However, based upon our understanding of the mechanisms associated with preconditioning, this has allowed us to revisit and focus on other cardioprotective strategies.

The novel phenomenon of postconditioning has attracted much attention over the past 2 years. Strategies such as postconditioning can protect the myocardium from the detrimental effects of ischaemia-reperfusion injury, and may be applied as adjunctive therapy to current reperfusion strategies. Some of the mechanisms involved in this novel approach have been discovered, most of which are shared by agents that protect the myocardium when given at reperfusion, and therefore, these may be considered as pharmacological postconditioning mimetics. Additionally, it now appears that both IPC and ischaemic postconditioning both share a common pathway that mediates their protective effects. However, taken together, current animal studies suggest that the effectiveness of postconditioning is dependent on several determinants such as duration of cycles, timing of application, cellular pathways involved, and that in themselves, these determinants appear to be species dependent.

Ischaemic postconditioning, in addition to the above direct active effects on the myocyte also reduces myocardial injury through the passive effects of modified reperfusion resulting in reduced endothelial dysfunction, myocardial stretch and hypercontracture, mitochondrial calcium overload, myocardial oedema, and reactive oxygen species which has been known for a number of years to be beneficial. The comment by Heusch that postconditioning is possibly "old wine in a new bottle" is very apt[131] and indicative that we have probably just re-discovered an old phenomenon. However, the use of the term postconditioning has initiated a number of studies which might not have otherwise been undertaken had this phenomenon not been given such an appealing name. These important studies have allowed us to gain a greater understanding of cellular pathophysiology and in themselves, created renewed interest which has enabled the concept of reperfusion injury to be revisited.

Chapter Two: OBJECTIVES AND HYPOTHESES

The main objectives of this study were threefold. The first was to determine whether the phenomenon of ischaemic postconditioning could protect isolated perfused rat hearts against myocardial ischaemia-reperfusion injury. At the time of this study, ischaemic postconditioning had only been demonstrated in two in vivo animal models and cardiomyocytes. However, whether pro-survival cellular signalling pathways were responsible for mediating the protection had not been demonstrated to date. The second aim was to determine the effect of ischaemic postconditioning in type 2 diabetic rat hearts, diabetes being a major risk factor for coronary artery disease, as all previous studies had focused on healthy hearts. If ischaemic postconditioning failed to protect the myocardium however, the third objective was to determine the effect of a well known cardio-protective strategy, namely IPC, in type 2 diabetic hearts and the cellular mechanisms involved.

2.1 Hypothesis One

Ischaemic postconditioning protects the healthy heart by activating the RISK pathway, specifically the PI3K-Akt pathway, in the first few minutes of reperfusion.

This hypothesis was determined as follows by:

1) Investigating whether ischaemic postconditioning protects the normal rat myocardium from ischaemia-reperfusion injury.

2) Determining the role of the pro-survival PI3K-Akt pathway in this protection.

3) Determining the involvement of the downstream mediators of the PI3K-Akt pathway, namely endothelial nitric oxide synthase (eNOS) and p70S6 kinase (p70S6K) in ischaemic postconditioning.

2.2 Hypothesis Two

Ischaemic postconditioning protects the type 2 diabetic rat heart against the effects of ischaemic-reperfusion injury.

This hypothesis was determined as follows by:

1) Investigating whether ischaemic postconditioning protects the type 2 diabetic rat myocardium from ischaemia-reperfusion injury and determining the role of the PI3K-Akt pathway in this phenomenon.

2.3 Hypothesis Three

Ischaemic preconditioning can protect the type 2 diabetic rat heart against the effects of ischaemic-reperfusion injury.

This hypothesis was determined as follows by:

1) Investigating whether IPC protects the type 2 diabetic myocardium from ischaemia-reperfusion injury.

2) Determining the level of IPC stimulus required to achieve this protection.

3) Determining the role of the pro-survival PI3K-Akt pathway in this protection.

Chapter Three: GENERAL METHODS

3.1 Animals

Male Sprague-Dawley and Wistar rats were obtained from Charles River UK Limited, (Margate, UK), and male Goto-Kakizaki rats were obtained from Taconic (Denmark). All animals received humane care in accordance with the Home Office *Guidance on the Operation of Animals (Scientific Procedures) Act 1986* (Her Majesty's Stationery Office, London, UK). All animals were fed a standard chow diet and had free access to fresh drinking water. Each animal cage housed a maximum of 4 rats and all animals were allowed to acclimatize for a minimum of 5 days upon receipt from the supplier before use. Housing conditions consisted of a 12 hour light-dark cycle, 19-22°C temperature maintenance and 55± 10% humidity.

3.2 In Vitro Perfused Rat Heart Model

3.2.1 Langendorff Isolated Heart Model

This study utilized the isolated perfused heart technique as described by Oscar Langendorff in 1895. The basic principle is to retrogradely perfuse under constant pressure or flow any fluid that sustains myocardial function via the ascending aorta. Retrograde perfusion into the aorta causes the aortic valve to close and the perfusate to flow down the coronary arteries to supply the myocardium. Through the coronary venous system, perfusate returns via the

coronary sinus into the right atrium and flows into the right ventricle. The perfusate is ejected from the right ventricle during cardiac systole into the opened pulmonary artery to flow along the surface of the heart.

3.2.1.1 Excision of the Heart

Rats were heparinized with sodium heparin (300IU, Multiparin CP Pharmaceuticals Ltd) to prevent coronary vasculature thrombosis and anaesthetized with sodium pentobarbital (50mg/kg, Sagatal-Rhone Merieux) intra-peritoneally. Adequate anaesthesia was determined by the cessation of the pedal withdrawal reflex to a painful stimulus before weighing each animal. Under deep anaesthesia, a transverse abdominal incision was performed to expose the diaphragm which was incised along its lateral margins to gain access to the thorax. Bilateral vertical incisions through the ribs were made along the thoracic cage and the anterior chest wall was retracted to allow easy access to the heart. Excision of the heart was achieved by reflecting the heart superiorly and a clean incision through the ascending aorta was made.

3.2.1.2 Isolated Heart Perfusion

After rapid excision, hearts were placed in ice-cold perfusion buffer to immediately reduce metabolic activity then mounted via the ascending aorta onto the aortic cannula of the Langendorff perfusion system. A diagrammatic representation of the Langendorff apparatus is shown in Figure 3.1 and digital images of the same in Figures 3.2a and b. With experience, the time taken to

excise the heart, to being mounted and perfused, was less than 1 minute. Rapid mounting of the heart after excision was necessary to avoid the initiation of ischaemic preconditioning. The hearts were perfused retrogradely via the aorta with a modified Krebs-Henseleit buffer prepared from chemicals supplied by BDH Laboratories (Merck Eurolab, UK) containing (mM): NaCl 118, $NaHCO_3$ 25, glucose 11, KCl 4.7, $MgSO_4.7H_20$ 1.22, KH_2PO_4 1.21 and $CaCl_2$ 1.84, filtered through 1µm Whatman microfilters. Perfusion pressure was determined by gravity and fixed at 85 mmHg. All solutions were bubbled with 95% O_2/5% CO_2 to maintain a pH of between 7.35-7.5 measured using a blood gas autoanalyzer (ABL 700, Radiometer, Copenhagen). The left atrial appendage was excised and a fluid filled latex balloon attached to a pressure transducer (Oxnard, CA) connected to a Multitrace 2 machine pen-recorder (Lectromed, Letchworth, UK). The balloon was inserted into the left ventricular cavity and inflated with water to give a left ventricular end-diastolic pressure of 5-10 mmHg. Temperature of the perfused heart was maintained between 36.5 and 37.5°C using a heated organ bath, warmed via a thermostat regulated waterpump. Additional heating was provided by lamp heat. Temperature was monitored by placement of a thermo-wire (Techne, Cambridge, UK) into the opened pulmonary artery and recorded with a digital temperature recorder (Digitron Instrumentation Ltd, Herts, UK). A 3/O silk suture was placed to pass under the left anterior descending coronary artery (LAD) and the ends passed through a pipette tip to form a snare. Cardiac function was monitored by measurements of rate/pressure product (RPP=heart rate x developed pressure), and coronary flow rate (CFR) throughout stabilization, ischaemia and reperfusion at frequent intervals. Coronary flow rate in this constant pressure

system is determined by the coronary artery vascular resistance which in turn is dependent on the arteriolar vascular smooth muscle tone and myocardial contractions. Coronary flow rate was measured by collecting the coronary effluent over one minute (ml/min) at frequent intervals. The rate pressure product (mmHg/min x 10^3) is the sum of the ventricular developed pressure multiplied by the heart rate. The developed pressure is the product of the maximal pressure generated in systole minus the resting diastolic pressure.

Figure 3.1: The Langendorff Perfusion System. Diagrammatic representation of the Langendorff perfusion apparatus for isolated rat heart studies. Warmed, oxygenated Krebs-Henseleit buffer is perfused under constant pressure into the isolated heart mounted onto the aortic cannula. The water-filled latex balloon placed in the left ventricle monitors the pressure generated by the cardiac contractions which is transferred to the pen recorder. The coronary effluent is collected as a measure of coronary flow rate.

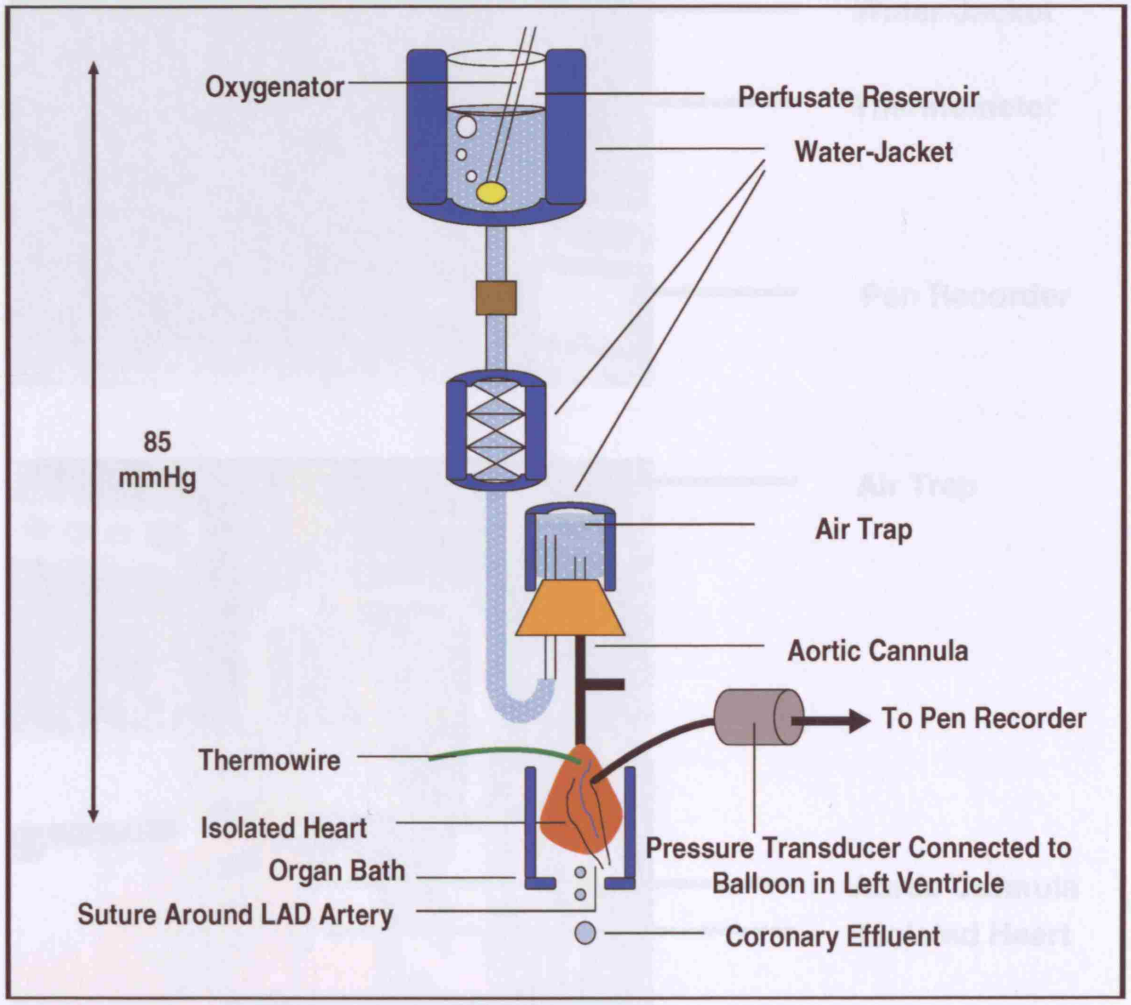
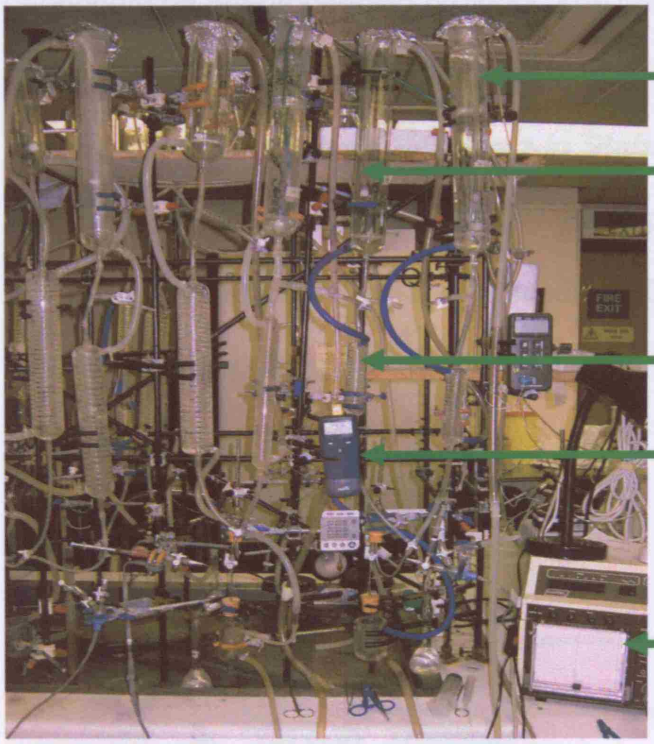


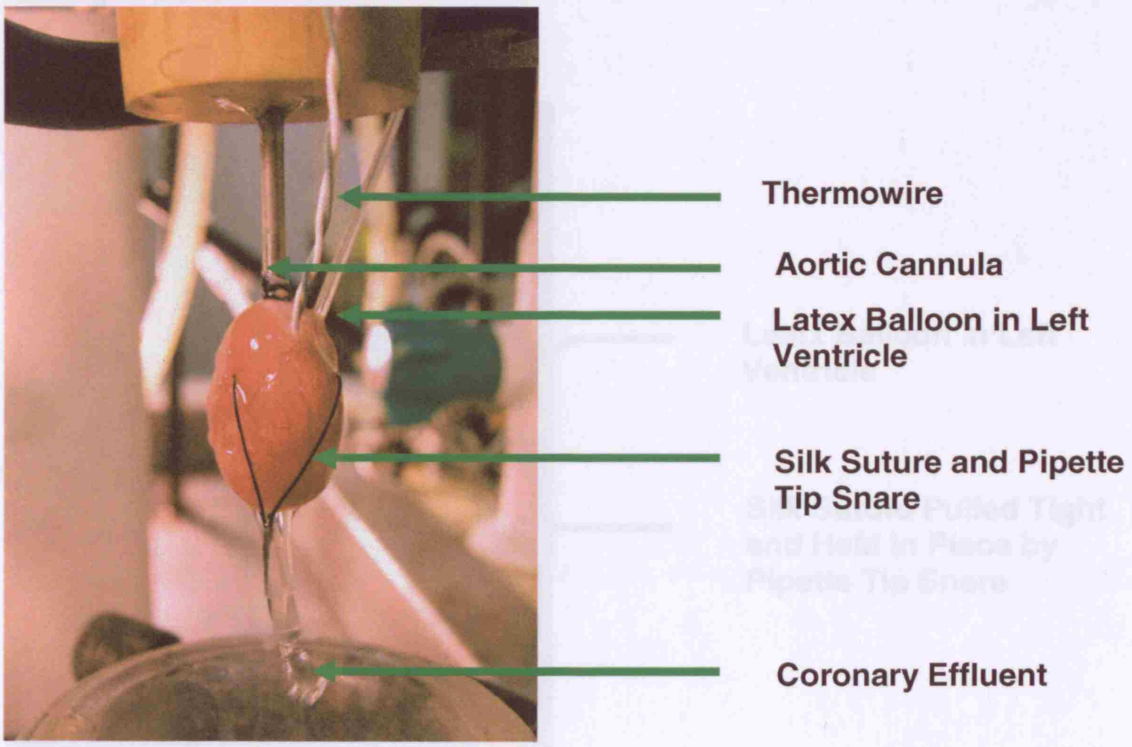
Figure 3.2a and b: The Langendorff Perfusion System. Digital images of the Langendorff perfusion apparatus.



3.2.1.2 *Induction of Ischaemic-Reperfusion Injury*

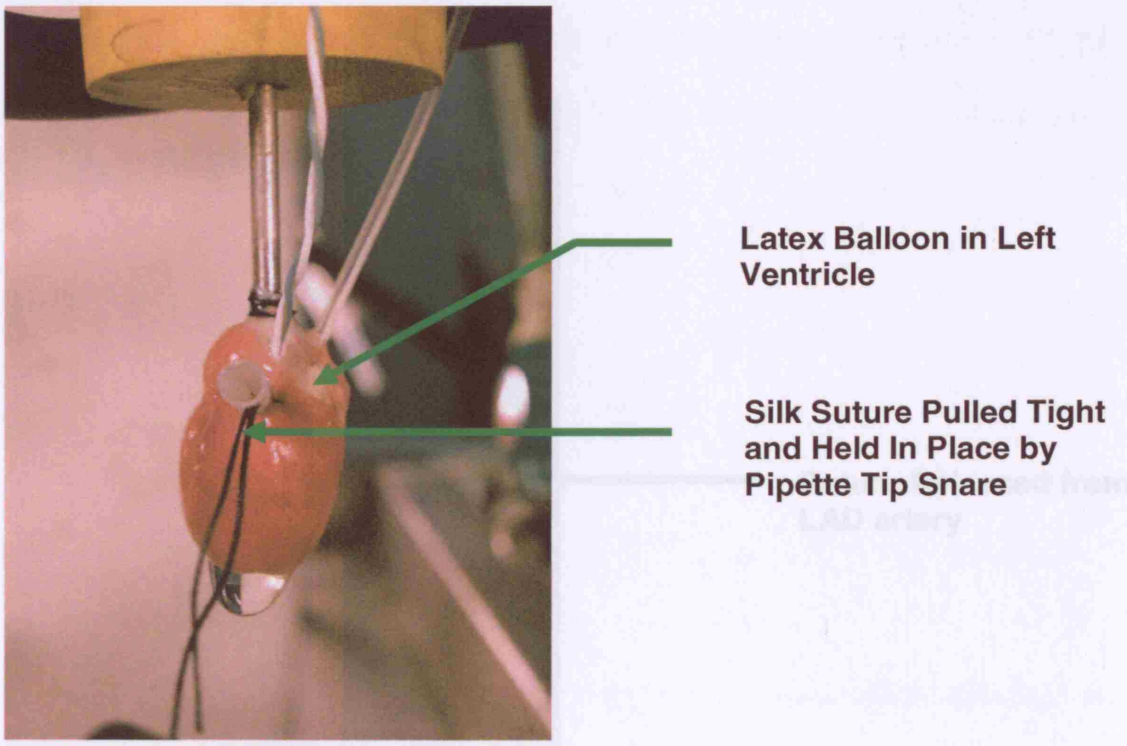
All hearts were allowed to stabilize (see Figure 3.3) for a minimum period dependent on the proceeding protocol (see later).

Figure 3.3: The Isolated Rat Heart in Stabilization. Image showing isolated heart with silk suture around LAD artery and pipette tip snare. The thermowire and ballon cannula can be seen in position. Coronary effluent is collected as it runs off the epicardial surface.



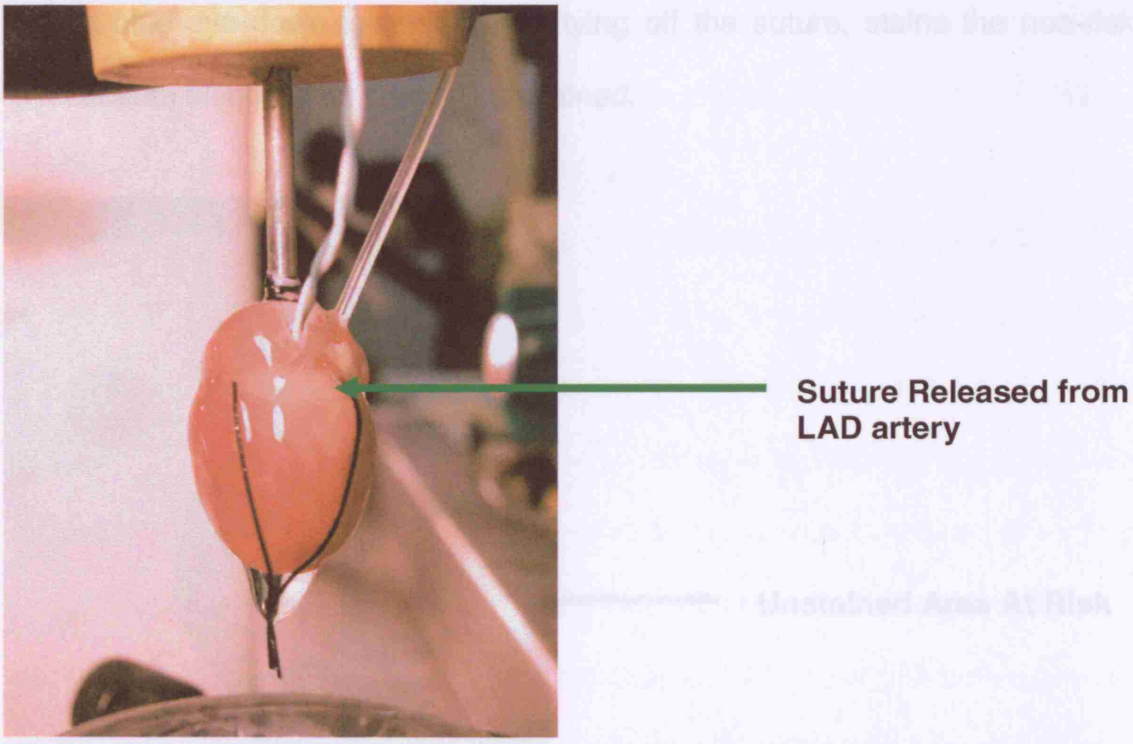
At the end of the stabilization period, all hearts were subjected to 35 minutes of regional ischaemia. This was achieved by pulling the silk suture tightly to occlude the LAD and locking in place with the pipette tip snare as shown in Figure 3.4. Sufficient occlusion of the LAD artery to achieve ischaemia was confirmed by an approximate 50% reduction in the coronary flow rate and a drop in rate pressure product (RPP).

Figure 3.4: The Isolated Rat Heart in Ischaemia. Image showing isolated heart with silk suture pulled tight around the LAD artery and pipette tip snare holding in place.



At the end of the 35 minute ischaemic period, reperfusion was achieved by releasing the pipette tip snare to free the suture as shown in Figure 3.5. Successful reflow down the LAD artery was confirmed by an increase in coronary flow and a gradual recovery in rate pressure product but not to pre-ischaemic values. However, throughout the 2 hour reperfusion period all hearts showed a gradual decline in coronary flow and RPP which is indicative of the "dying" ischaemic-reperfusion protocol.

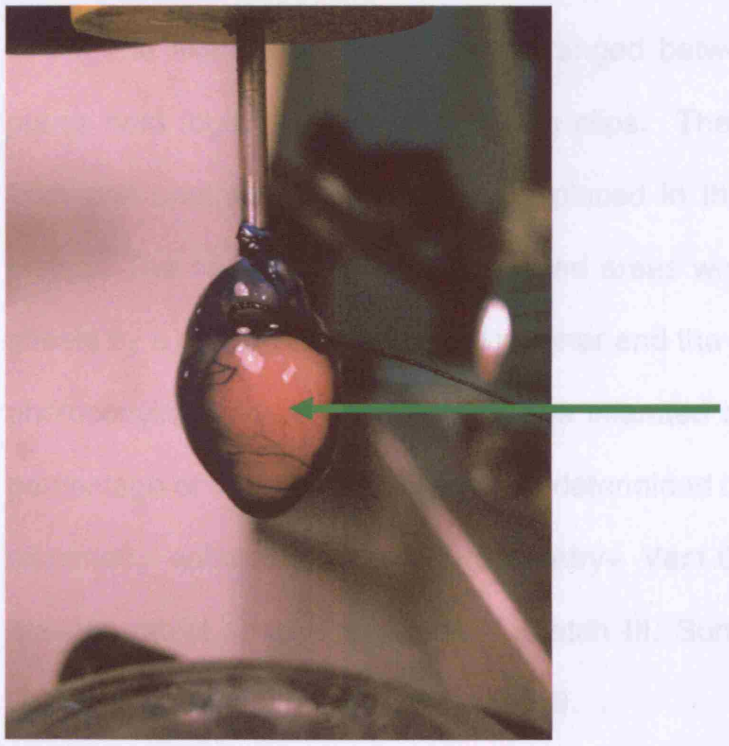
Figure 3.5: The Isolated Rat Heart in Reperfusion. Image showing isolated heart with suture released. The evolution of the myocardial infarct can be seen appearing as the white area indicated by the arrow.



3.2.1.3 *Myocardial Infarct Evaluation*

The volume of myocardium supplied by the occluded coronary artery is referred to as the "area at risk" (AAR), since it is this area that is at risk of infarction. In order to visualize this AAR it is necessary to delineate the non-risk myocardium from the AAR and this is achieved at the end of reperfusion by tying off the silk suture to ligate the coronary artery once more. Approximately 2 mls of 0.25% Evan's blue dye (Sigma Chemicals, Poole, UK) was injected into the heart via the side port of the aortic cannula to stain the non-risk myocardium blue and leaving the remaining AAR unstained as shown in Figure 3.6.

Figure 3.6: AAR Delineation in the Isolated Rat Heart. Injection of 0.25% Evan's blue dye down the aorta after tying off the suture, stains the non-risk myocardium blue leaving the AAR unstained.



Once stained, the hearts were removed from the aortic cannula and weighed before being frozen at -20°C for a minimum of 4 hours. Hearts were then sliced into five 2 mm slices from the base of the suture to the apex of the heart in preparation for infarct determination by the triphenyltetrazolium chloride (TTC) staining method[132]. Using this technique, NADH enzymes and other cofactors present in viable myocardial tissue, reduces TTC to form the red pigment formazan. Infarcted myocardial tissue loses these dehydrogenase enzymes during the reperfusion phase and does not form the formazan pigment when incubated with TTC and therefore remains unstained and is white in appearance. Heart slices were then incubated at 37°C in a 1% solution of TTC/phosphate buffer, pH 7.4 (Sigma Chemicals, Poole, UK) for 12 minutes, before being placed in a 10% formalin solution (BDH Laboratory Supplies, Poole, UK) at room temperature for a minimum period of 16 hours. In addition to fixing the slices, this helps to improve the contrast between the red stained viable and white infarcted myocardial tissue.

The fixed heart slices were arranged between two colourless Perspex plates held together by bulldog spring clips. The two plates were separated from one another by 2 mm spacers placed in the four corners between the plates. The non-risk, AAR and infarcted areas were traced onto clear acetate sheets by a colleague in a blinded manner and the tracings increased in size by photocopying from A4 to A3 size. The infarcted myocardium expressed as a percentage of the AAR (I/R zone) was determined by the use of a computerized planimetry software program (Planimetry+ Ver1.0, copyright Traasdahl) and drawing tablet package (Summa Sketch III, Summagraphics, Seymour, CT, USA) as illustrated in Figure 3.7 and 3.8.

Figure 3.7: TTC Stained and Fixed Heart Slices. Control heart after 35 minutes ischaemia and 2 hours reperfusion demonstrating infarcted (white), AAR (red) and non-risk (blue) areas.

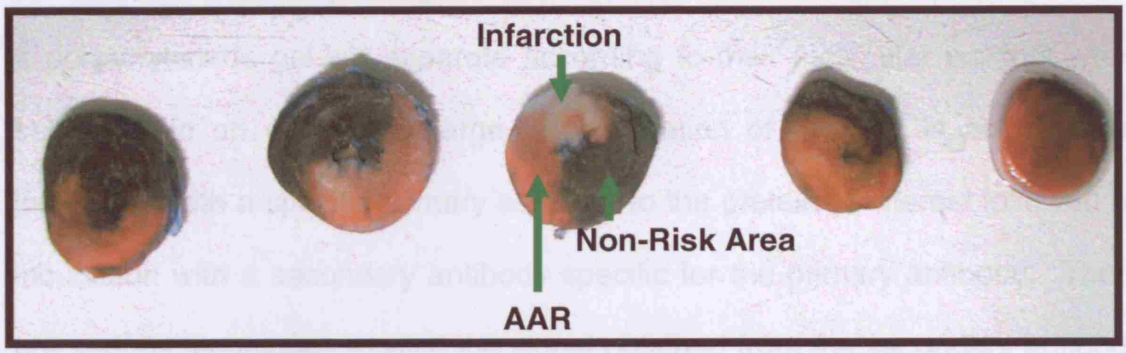
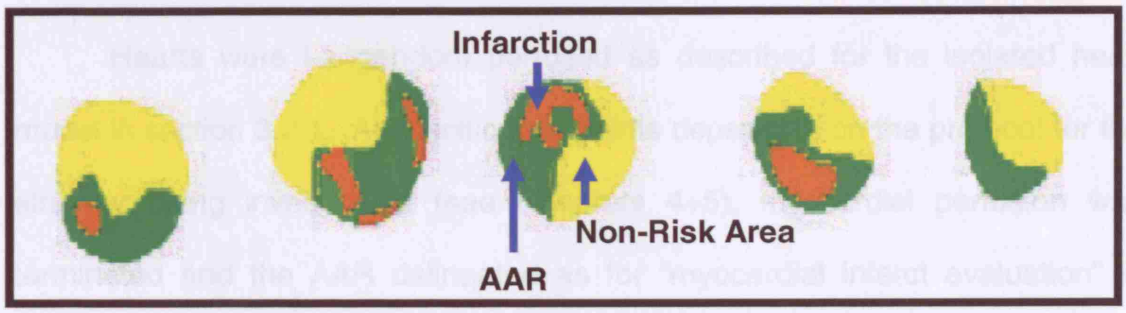


Figure 3.8: Computerized Planimetry of Heart Slices. The above control heart planimetered demonstrating infarcted (red), AAR (green) and non-risk (yellow) areas.



3.2.1.4 Exclusion Criteria for Isolated Hearts

Hearts were excluded from the study if: 1) after stabilization RPP was less than17 mmHg/min, 2) after stabilization coronary flow rate was less than 12 mls/min, 3) planimetered AAR was less than 0.3 cm^3 or greater than 0.75 cm^3, 4) I/R zone was less than 5% or greater than 90%.

3.3 Western Blot Analysis

Detection of proteins using Western blot analysis relies on the fact that proteins are electrically charged complexes. A mixture of proteins placed onto a polyacrylamide gel will separate according to their molecular weights when subjected to an electrical charge. Identification of proteins is achieved by incubation with a specific primary antibody to the protein of interest followed by incubation with a secondary antibody specific for the primary antibody. There are various techniques to elicit the signal obtained from the secondary antibody and in the case of this study the horse radish peroxidase (HRP) technique will be described later.

3.3.1 Collection of Myocardial Tissue Samples

Hearts were Langendorff perfused as described for the isolated heart model in section 3.2.1. At specific time points depending on the protocol for the strategy being investigated (see Chapters 4+5), myocardial perfusion was terminated and the AAR delineated as for "myocardial infarct evaluation" in section 3.2.1.3. However, after staining with 0.25% Evan's blue dye, the AAR ventricular tissue was rapidly excised and immediately snap frozen in liquid nitrogen between pre-cooled tissue clamps. The frozen sample was then broken into several pieces before being stored at -80°C.

3.3.2 Sample Protein Extraction

For each sample, approximately 50mg of frozen tissue was homogenized on ice using a Polytron model T25 homogeniser (IKA Labortechnik T25, Janke & Kunkel GmbH & Co., Germany), in 300 µl suspension buffer consisting (mmol/l): NaCl 100, TRIS 10 (pH 7.6), EDTA 1 (pH 8.0), sodium pyrophosphate 2, sodium fluoride 2, β-glycerophosphate 2, phenyl methyl sulphonyl fluoride (PMSF) 0.1 µg/ml, and 1 µg/ml each of trypsin inhibitor, leupeptin, aprotonin, and protease inhibitor. The suspension buffer contains both lytic agents to extract proteins from the cells and protease inhibitors to prevent degradation of these proteins. The sample homogenate was then centrifuged at 10,000 rpm for 10 minutes at 4°C which separates the homogenate into a protein rich supernatant and cellular debris pellet. Aliquots of 260 µl of supernatant were drawn off, kept cool on ice and divided into 2x5 µl samples for protein quantification and the remaining 250 µl supernatant was diluted in equal 250 µl volumes of sample buffer containing (mmol/l): Tris 100 (pH 6.8), sodium dodecylsulphate (SDS) 2%, dithiothreitol (DTT) 200, glycerol 20%, and bromophenol blue 0.2%. Each supernatant/sample buffer mixture was heated at 100°C for 10 minutes prior to being stored at -80°C for later analysis.

3.3.3 Protein Quantification

Each sample was analyzed for protein content using a bicinchoninic acid-based (BCA™) protein assay reagent system (Pierce, Rockford, USA). This assay depends on the reduction of ionized copper which results in the production of a purple coloured BCA-copper complex which is subsequently measured at an optical wavelength of 562 nm using a spectro-photometer (Janway model 6405 UV/Vis, Dunmow, UK).

A standard curve using increasing concentrations of 0, 10, 20, 30, 40 µg/µl of bovine serum albumin (BSA) was produced. Since the association between the measured absorbance and protein concentration bears a linear relationship from 20-2000 µg/ml, the protein concentration (µg/µl) in each test sample can be obtained by plotting the absorbance on the standard curve, thus ensuring equal protein loading of the polyacrylamide gel.

3.3.4 Gel Preparation

For the Western blot studies, sodium dodecylsulphate - polyacrylamide (SDS-PAGE) gels were prepared. Each gel mixture was introduced between two alcohol cleaned glass plates separated by spacers loaded onto a gel loading self sealing system. Each SDS-PAGE gel sheet consisted of a lower 12.5% running gel on top of which was an upper 5% stacking gel. The running gel comprised of 12 ml distilled water, 9 ml running gel base (containing 1.5M TRIS, 0.4% SDS in distilled water, adjusted with HCl to pH 8.8), 15 ml 30% acrylamide, 40 µl TEMED and 200 µl 10% ammonium persulphate (APS). The stacking gel comprised of 7.0 ml distilled water, 3 ml stacking gel base

(containing 0.5 M TRIS, 0.4% SDS in distilled water, adjusted with HCl to pH 6.8), 2 ml 30% acrylamide, 30 µl 8% bromophenol blue, 24 µl TEMED and 120 µl 10% ammonium persulphate. Wells were formed in the stacking gel by the placement of a 24 pronged comb between the top of the glass plates prior to the introduction of the stacking gel.

3.3.5 Electrophoresis Technique

30 µg of protein sample prepared in section 3.3.2 was pipetted into each well in addition to 10 µl of a dual colour molecular weight electrophoresis marker (BioRad, UK) into the first well. The gel plates were then loaded into a water cooled electrophoresis system with the top and bottom end of the plates in contact with running buffer comprising of glycine 14.4 g/l, SDS 1.0 g/l, Tris 3.0, 1 litre distilled water, and the gels were allowed to run at 120 volts constant for approximately 4 hours.

3.3.6 Protein Transfer

At the end of electrophoresis, the gels were removed and carefully trimmed to size before being placed on a Hybond ECL nitrocellulose membrane (Amersham, UK) which was cut to the same size as the gel. The gel and membrane were then sandwiched between two large pieces of Whatman filter paper and gently smoothed to expel any trapped air bubbles. The whole gel sandwich was then mounted in a transfer rack and placed vertically into a transfer tank system filled with transfer buffer comprising 200 ml methanol, 700

ml distilled water and 100 ml of 10x strength transfer buffer concentrate (containing TRIS 3.0 g/l, glycine 14.4 g/l, and 1 litre distilled water). The transfer system was then run at 140 mA constant overnight for a minimum of 16 hours.

The following morning, the membranes were removed and stained with Ponceau Red (Sigma Chemicals, Poole, UK) to ensure adequate transfer of proteins from the gels.

3.3.7 Immunoblotting

Before antibody probing for proteins, the membranes were placed in trays on a rocking platform at room temperature and washed for 5 minutes in washing buffer containing (Na_2HPO_4 1.6 g/l, NaCl 8.0 g/l,1 litre distilled water, Tween-20 1ml, adjusted with HCl to pH 7.2). In order to reduce any interference signal from non-specific binding of antibody to background proteins, membranes were incubated for 1 hour in 5% blocking buffer containing (100 ml washing buffer and 5 g powdered milk).

Membranes were then washed a further 3 times for 5 minutes in washing buffer then incubated with primary antibody (raised in rabbit) specific for the protein of interest for a minimum of 2 hours at room temperature. Each primary antibody was prepared as a 1:1000 dilution in a 5% BSA solution containing (100 ml washing buffer and 5 g BSA).

After decanting the excess primary antibody solution, the membranes were washed 3 times for 5 minutes in washing buffer then incubated with anti-rabbit secondary antibody conjugated to horse-radish peroxidase, again at a

1:1000 dilution in a 5% milk solution containing (100 ml washing buffer and 5 g powdered milk) for 1 hour.

The membranes were washed a further 3 times for 5 minutes in washing buffer. Protein detection was achieved by incubation of the membranes for 2 minutes with enhanced chemi-luminescence (ECL) Western blotting detection reagent (Amersham, UK) to elicit the horse-radish peroxidase signal.

3.3.8 Protein Band Quantification

Visualization of the protein bands on the nitrocellulose membranes was achieved by exposure of the membranes against photographic Kodak AR film and subsequent incubation of the film in Kodak developing and fixing reagents (Sigma Chemicals, Poole, UK).

After drying, the films were digitally scanned using a flat-bed image scanner and saved to disc. Densitometry of the protein bands was analyzed using a National Institutes of Health (NIH) software program (ver 1.63). The relative densitometries of individual phosphorylated protein bands were normalized against their respective total protein bands and the results expressed as a percentage in arbitrary units (AU). Equal loading of the membranes with protein was confirmed by immunoblotting for a stable cellular protein such as β-actin (antibodies supplied by Abcam Ltd, UK) and the densitometries of the protein bands subsequently adjusted to account for unequal loading if necessary.

3.4 Statistical Analysis

Study data was analyzed using Statview statistical software (ver 4.5, Abacus Concepts Inc). Data was expressed as group means ± standard error of the mean (SEM). Between group comparisons were analyzed using one way analysis of variance (ANOVA) and Fisher's protected least significance difference (PLSD) post hoc test. Cardiac functional data was analyzed using repeated-measures ANOVA with Fisher's PLSD where significance was found. Differences were considered statistically significant when $p < 0.05$.

Chapter Four: MECHANISMS OF ISCHAEMIC POSTCONDITIONING IN NORMAL HEARTS

4.1 Introduction

The protection afforded by the phenomenon of IPC, in which short periods of ischaemia protect the myocardium against a subsequent lethal ischaemic insult, can only be utilized if the IPC mimetic is applied prior to the index ischaemic episode[8]. This is problematical in the clinical setting of ischaemia-reperfusion where for the most part, the onset of the index ischaemic episode, such as the acute myocardial infarction, is unpredictable. Therefore, the ability to protect the heart against ischaemia-reperfusion injury by intervening at the time of reperfusion, provides an approach which is both feasible and under the control of the operator.

In this context, the recently described phenomenon of ischaemic post-conditioning, first coined by Vinten-Johansen's group[110], in which brief intermittent repetitive interruptions to reperfusion, at the immediate onset of reperfusion after a prolonged period of ischaemia reduced myocardial injury to an extent comparable with IPC, and offers a novel approach to myocardial protection. Suggested mechanisms of protection include: (1) a reduction in neutrophil accumulation[110]; (2) attenuation of oxidative stress[114]; (3) improved endothelial dysfunction; (4) a reduction in apoptotic cell death[110]; and (5) attenuation of mitochondrial calcium accumulation[115].

Activation of the pro-survival kinases phosphatidylinositol 3-OH kinase (PI3K)-Akt, and the mitogen activated protein kinase (MAPK), p42/p44 extra-cellular signal-regulated kinases (ERK1/2) at the time of reperfusion, which together comprise the Reperfusion Injury Salvage Kinase (RISK) pathway, following an episode of lethal ischaemia, is cardio-protective[100]. In this chapter we test the hypothesis that ischaemic postconditioning protects the heart by activating the RISK pathway, specifically the PI3K-Akt pathway in the first few minutes of reperfusion.

4.2 Hypothesis One

Ischaemic postconditioning protects the healthy heart by activating the RISK pathway, specifically the PI3K-Akt pathway, in the first few minutes of reperfusion.

4.3 Objective One

To investigate whether ischaemic postconditioning protects the normal rat myocardium from ischaemia-reperfusion injury.

4.3.1 Animals

Male Sprague-Dawley rats (270 to 450g, n=44) were used for this investigation. The hearts were excised and Langendorff perfused as described in section 3.2.1. All hearts were subjected to 35 minutes regional ischaemia and 2 hours reperfusion before infarct analysis by TTC staining.

4.3.2 Experimental Protocols

The experimental protocols used in these studies are depicted in Figure 4.1. Hearts were randomly assigned to one of the following groups:

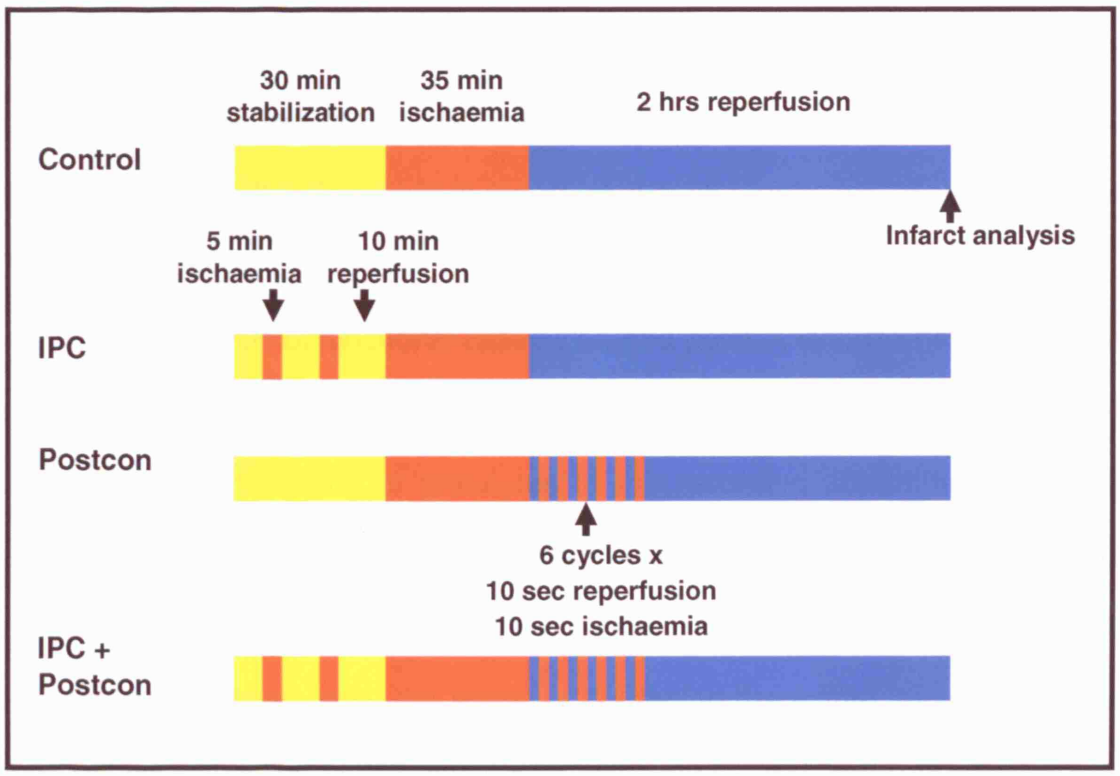
1) **Controls** (n=11) subjected to 35 minutes regional ischaemia and 2 hours reperfusion only;

2) **Ischaemic Preconditioning** (n=11) comprising 2 cycles of 5 minutes global ischaemia followed by 10 minutes reperfusion, prior to the index ischaemia;

3) **Postconditioning** (n=10) comprising 6 cycles of 10 seconds reperfusion followed by 10 seconds global ischaemia following the index ischaemia at the immediate onset of reperfusion;

4) **Ischaemic Preconditioning and Postconditioning** (n=7) comprising a combination of both the protocols for IPC and postconditioning.

Figure 4.1: Experimental Protocols for Investigating Ischaemic Postconditioning in Normal Hearts. IPC - ischaemic preconditioning, Postcon - postconditioning.



4.3.3 Results

4.3.3.1 Exclusions

A total of 44 animals were used for this part of the study of which 5 were excluded due to the criteria as described in section 3.2.1.4.

4.3.3.2 Cardiac Functional Data

There were no significant differences between groups with respect to either body weight or AAR (see Table 4.1). At the end of the stabilization period, coronary flow and RPP were comparable between all groups with no significant differences. Similarly, as expected coronary flow and RPP were reduced after the induction of ischaemia to a similar degree and recovered to the same extent after the onset of reperfusion in all groups (see Figure 4.2 A+B, Table 4.2 and 4.3).

The analysis of functional data as an end point in isolated heart studies is unreliable and inaccurate. Measurement of functional data requires reproducible transduction of pressures to the recording apparatus. The size and position of the latex balloon in the left ventricle is critical in determining this reproducibility since pressures will vary in different locations within the heart. At the induction of ischaemia, the action of tightening the suture invariably moves the balloon within the ventricular cavity when compared to the position during stabilization and moves again with the release of the suture in reperfusion. For this reason, coronary flow and RPP are only reliable parameters within the

periods of stabilization, ischaemia and reperfusion themselves and not reliable for comparison between these periods. In this manner, a reduction in coronary flow and RPP was used to indicate the adequate onset of ischaemia and reperfusion. Further support against the use of cardiac function came from the observation that those hearts with poor function at the end of reperfusion did not necessarily have larger infarcts than those with better function.

Table 4.1: Animal Characteristics of Experimental Groups. Values are mean ± SEM. IPC - ischaemic preconditioning, Postcon - postconditioning

Group	Number	Body Weight (g)	Risk Volume (cm^3)
Control	11	338.7 ± 11.2	0.504 ± 0.025
IPC	11	349.3 ± 8.2	0.558 ± 0.032
Postcon	10	333.6 ± 8.1	0.556 ± 0.031
IPC+Postcon	7	379.1 ± 10.0	0.536 ± 0.028

Figure 4.2: Cardiac Functional Data. Coronary flow rate and RPP changes throughout the ischaemic-reperfusion protocol. Points represent mean values, standard error bars have been removed for clarity. S – stabilization, I – ischaemia, R – reperfusion. IPC - ischaemic preconditioning, Postcon - postconditioning

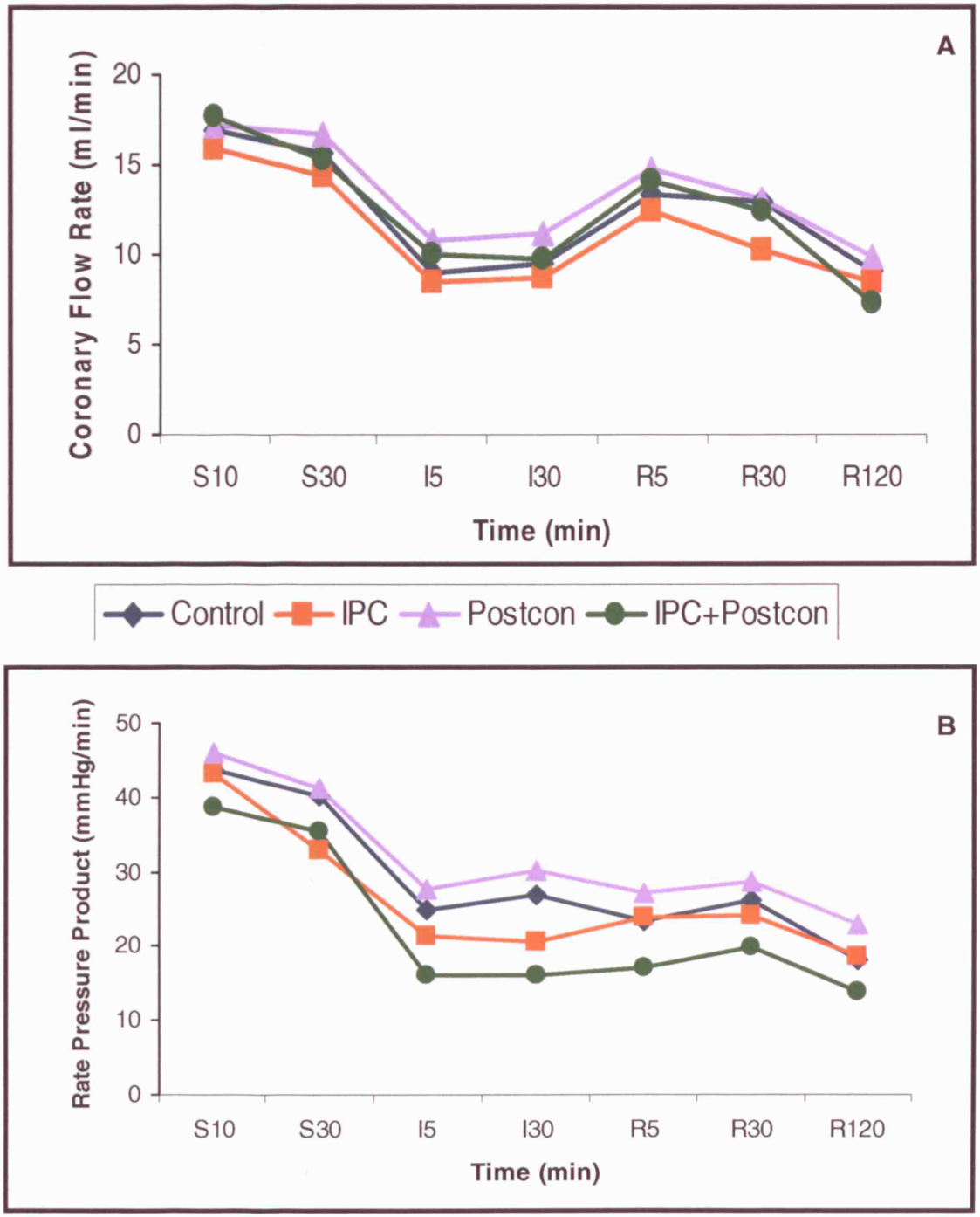


Table 4.2: Coronary Flow Rate in Experimental Groups (ml/min). Values are mean ± SEM. * p <0.05 compared with control.

S – stabilization, I – ischaemia, R – reperfusion, IPC - ischaemic preconditioning, Postcon - postconditioning

Group	S 10 min	S 30 min	I 5 min	I 30 min	R 5 min	R 30 min	R 120 min
Control	16.9 ± 1.2	15.6 ± 1.3	9.0 ± 0.8	9.5 ± 0.8	13.3 ± 1.2	12.9 ± 1.0	9.1 ± 0.8
IPC	15.9 ± 1.0	14.4 ± 0.6	8.5 ± 0.5	8.7 ± 1.1	12.4 ± 0.6	10.2 ± 0.7*	8.5 ± 0.6
Postcon	17.2 ± 0.9	16.7 ± 1.7	10.8 ± 0.7	11.1 ± 0.6	14.8 ± 0.6	13.1 ± 0.6	9.9 ± 0.8
IPC+Postcon	17.7 ± 1.6	15.3 ± 0.8	10 ± 2.1	9.7 ± 2.2	14.1 ± 2.1	12.5 ± 1.0	7.3 ± 0.7

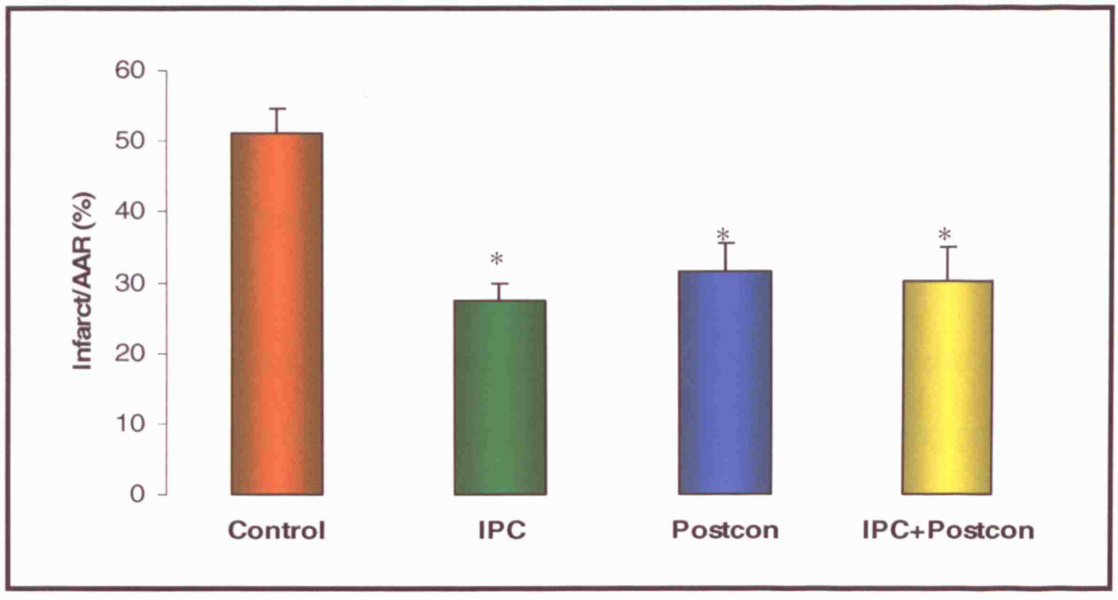
Table 4.3: Rate Pressure Product in Experimental Groups (mm Hg/min x 10^3). Values are mean ± SEM. * p <0.05 compared with control. S – stabilization, I – ischaemia, R – reperfusion, IPC - ischaemic preconditioning, Postcon - postconditioning

Group	S 10 min	S 30 min	I 5 min	I 30 min	R 5 min	R 30 min	R 120 min
Control	43.6 ± 2.8	40.3 ± 3.4	24.9 ± 2.0	26.9 ± 1.7	23.3 ± 2.1	26.2 ± 2.6	18.2 ± 1.9
IPC	43.2 ± 2.3	33 ± 2.4	21.3 ± 1.6	20.6 ± 2.7	23.8 ±1.7	24.1 ± 2.0	18.5 ± 1.8
Postcon	46 ± 5.1	41.3 ± 3.6	27.6 ± 2.5	30.2 ± 2.8	27.1 ± 3.1	28.7 ± 2.6	22.8 ± 3.0
IPC+Postcon	38.7 ± 3.9	35.4 ± 3.9	16.1 ± 1.3*	16 ± 2.6*	17 ± 1.7	19.9 ± 1.9	13.7 ± 1.8

4.3.3.3 Infarct Size

Infarct size, represented as a percentage of the area at risk (AAR), was significantly reduced in the IPC group (control 51.2 ± 3.4% vs IPC 27.5 ± 2.3%, $p<0.01$). Postconditioning in a similar manner reduced infarct size (control 51.2 ± 3.4% vs Postcon 31.5 ± 4.1%, $p<0.01$), an effect comparable to that of IPC. However, the protection afforded by the combination of both IPC applied before the index ischaemia, together with postconditioning at the onset of reperfusion was not additive, and did not differ compared with either strategy alone (IPC+Postcon 30.1 ± 4.8% vs 27.5 ± 2.3% with IPC and 31.5 ± 4.1% with Postcon, p=NS) as shown in Figure 4.3.

Figure 4.3: Ischaemic Postconditioning Protects the Isolated Heart from Ischaemic-Reperfusion Injury . The reduction in infarct size afforded by ischaemic postconditioning was comparable with that of IPC however, the combination of IPC and postconditioning was not additive. Values are mean ± SEM. * $p<0.05$. IPC - ischaemic preconditioning, Postcon - postconditioning



4.4 Objective Two

To determine the role of the pro-survival PI3K-Akt pathway in postconditioning-induced protection.

4.4.1 Animals

Male Sprague-Dawley rats (270 to 450g, n=40) were used for this investigation. The hearts were excised and Langendorff perfused as described in section 3.2.1. All hearts were subjected to 35 minutes regional ischaemia and 2 hours reperfusion before infarct analysis by TTC staining.

4.4.2 Chemicals

Wortmannin (Wort, Tocris, Bristol, UK) and LY294002 (LY, Tocris, Bristol, UK) were dissolved in the solvent dimethylsulphoxide (DMSO, Sigma Chemicals, Poole, UK) to give a final concentration when added to the Krebs Henseleit buffer of less than 0.02% which did not have any effect on infarct size or kinase phosphorylation.

4.4.3 Experimental Protocols

The experimental protocols used in this part of the study are depicted in Figure 4.4. Hearts were randomly assigned to one of the following groups:

1) **Control+DMSO** (n=4), control hearts with 0.02% DMSO given for the first 15 minutes reperfusion.

2) **Postconditioning+LY** (n=13), hearts with the specific PI3K inhibitor LY294002 (LY, 15μmol/l)[46] given for the first 15 minutes of reperfusion;

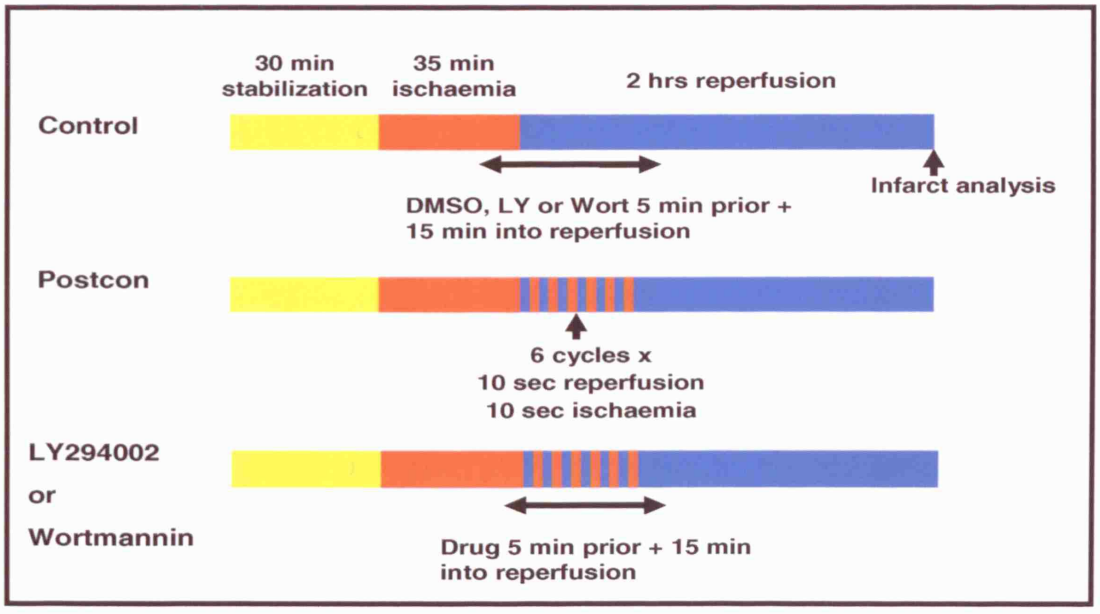
3) **Control+LY** (n=5), control hearts with LY given for the first 15 minutes of reperfusion;

4) **Postconditioning+Wort** (n=7) with the PI3K inhibitor Wortmannin (100nmol/l)[46] given for the first 15 minutes of reperfusion;

5) **Control+Wort** (n=5), hearts with Wortmannin given for the first 15 minutes of reperfusion.

Control and Postconditioning hearts were the same hearts as in section 4.3.2 and have been included here for comparison.

Figure 4.4: Experimental Protocols for Investigating the Role of the PI3K-Akt pathway in Postconditioning-induced Protection. Postcon - postconditioning, DMSO - dimethylsulphoxide, LY - LY294002, Wort - Wortmannin.



4.4.4 Results

4.4.4.1 Exclusions

A total of 40 animals were used for this part of the study of which 6 were excluded due to the criteria as described in section 3.2.1.4.

4.4.4.2 Cardiac Functional Data

Body weights and AAR were similar between experimental groups (see Table 4.4). At the end of the stabilization period, coronary flow and RPP were comparable between all groups with no significant differences. Similarly, as expected coronary flow and RPP were reduced after the induction of ischaemia to a similar degree and recovered to the same extent after the onset of reperfusion in all groups (see Figure 4.5 A+B, Table 4.5 and 4.6).

Table 4.4: Animal Characteristics of Experimental Groups. Values are mean ± SEM. * p <0.05 compared with control+DMSO. Postcon - postconditioning, DMSO - dimethylsulphoxide, LY - LY294002, Wort - Wortmannin

Group	Number	Body Weight (g)	Risk Volume (cm^3)
Control+DMSO	4	383.3 ± 14.7	0.521 ± 0.040
Postcon+LY	13	358.8 ± 14.3	0.478 ± 0.030
Control+LY	5	357.0 ± 28.2	0.528 ± 0.05
Postcon+Wort	7	422.9 ± 12.3	0.460.4 ± 0.037
Control+Wort	5	441.0 ± 9.0	0.554.8 ± 0.032

Figure 4.5: Cardiac Functional Data. Coronary flow rate and RPP changes throughout the ischaemic-reperfusion protocol. Points represent mean values, standard error bars have been removed for clarity. S – stabilization, I – ischaemia, R – reperfusion.

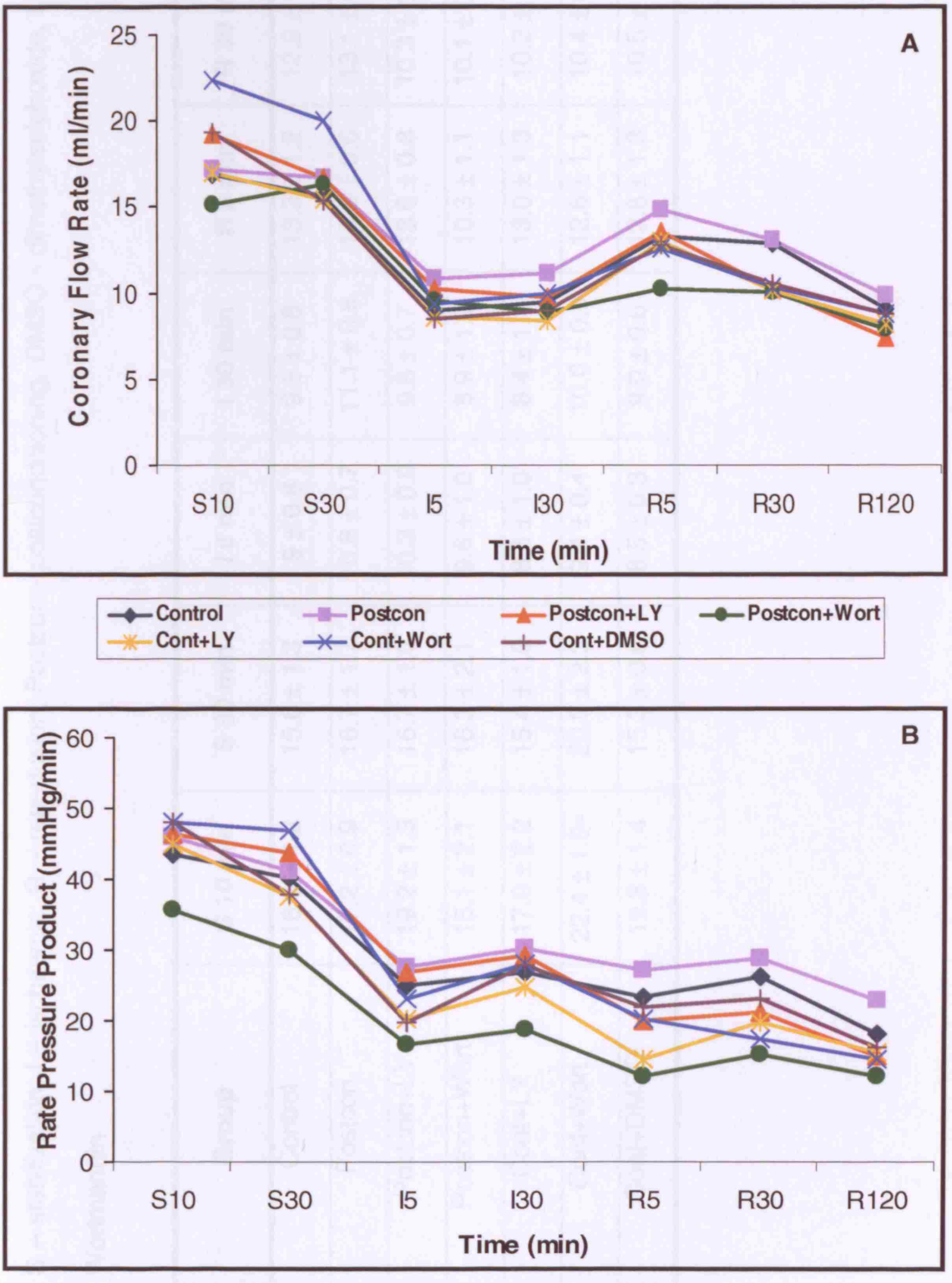


Table 4.5: Coronary Flow Rate in Experimental Groups (ml/min). Values are mean ± SEM. * p <0.05 compared with control. S – stabilization, I – ischaemia, R – reperfusion, Postcon - postconditioning, DMSO - dimethylsulphoxide, LY - LY294002, Wort - Wortmannin

Group	S 10 min	S 30 min	I 5 min	I 30 min	R 5 min	R 30 min	R 120 min
Control	16.9 ± 1.2	15.6 ± 1.3	9 ± 0.8	9.5 ± 0.8	13.3 ± 1.2	12.9 ± 1.0	9.1 ± 0.8
Postcon	17.2 ± 0.9	16.7 ± 1.7	10.8 ± 0.7	11.1 ± 0.6	14.8 ± 0.6	13.1 ± 0.6	9.9 ± 0.8
Postcon+LY	19.2 ± 1.3	16.7 ± 1.0	10.3 ± 0.9	9.8 ± 0.7	13.6 ± 0.8	10.3 ± 1.0*	7.4 ± 0.7
Postcon+Wort	15.1 ± 2.1	16.3 ± 2.1	9.6 ± 1.0	8.9 ± 1.2	10.3 ± 1.1	10.1 ± 0.9*	7.9 ± 0.8
Cont+LY	17.0 ± 2.2	15.4 ± 1.5	8.6 ± 1.0	8.4 ± 1.1	13.0 ± 1.3	10.2 ± 1.3	8.2 ± 1.0
Cont+Wort	22.4 ± 1.9*	20.0 ± 2.3	9.4 ± 0.4	10.0 ± 0.9	12.6 ± 1.1	10.4 ± 0.9	8.8 ± 0.6
Cont+DMSO	19.3 ± 1.4	15.3 ± 0.8	8.5 ± 0.3	9.0 ± 0.6	12.8 ± 1.3	10.5 ± 1.0	8.8 ± 1.1

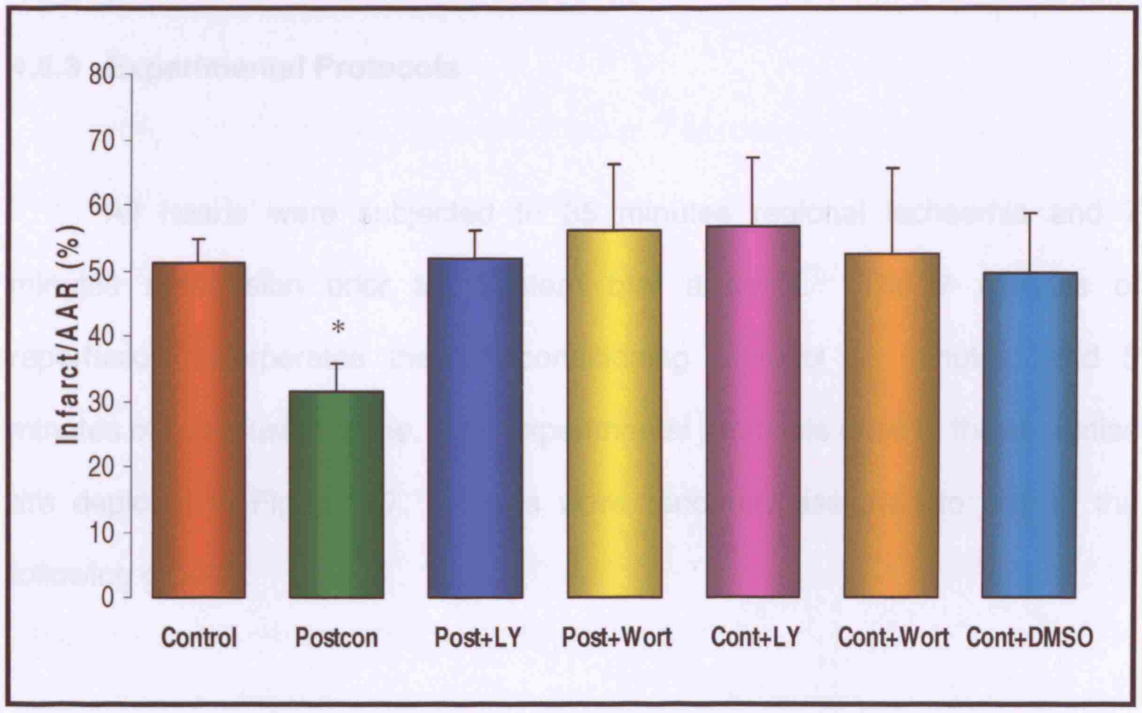
Table 4.6: Rate Pressure Product in Experimental Groups (mm Hg/min x 10^3). Values are mean ± SEM. * p <0.05 compared with control. S – stabilization, I – ischaemia, R – reperfusion, Postcon - postconditioning, DMSO - dimethylsulphoxide, LY - LY294002, Wort - Wortmannin

Group	S 10 min	S 30 min	I 5 min	I 30 min	R 5 min	R 30 min	R 120 min
Control	43.6 ± 2.8	40.3 ± 3.4	24.9 ± 2.0	26.9 ± 1.7	23.3 ± 2.1	26.2 ± 2.6	18.2 ± 1.9
Postcon	46.0 ± 5.1	41.3 ± 3.6	27.6 ± 2.5	30.2 ± 2.8	27.1 ± 3.1	28.7 ± 2.6	22.8 ± 3.0
Postcon+LY	46.5 ± 3.2	43.8 ± 3.3	27.0 ± 1.7	29.3 ± 1.9	19.9 ± 1.4	21.1 ± 2.2	14.9 ± 1.4
Postcon+Wort	35.7 ± 6.4	30.1 ± 5.0	16.7 ± 2.8*	18.8 ± 3.2*	12.2 ± 3.0*	15.2 ± 3.4*	12.1 ± 2.9
Cont+LY	45.0 ± 4.4	37.7 ± 3.6	20.2 ± 1.5	24.7 ± 2.9	14.5 ± 3.4*	19.7 ± 3.4	15.5 ± 2.6
Cont+Wort	48.2 ± 3.9	46.8 ± 5.8	23.0 ± 3.8	28.1 ± 5.6	20.3 ± 2.9	17.5 ± 3.9*	14.5 ± 2.4
Cont+DMSO	48.2 ± 4.2	37.9 ± 3.5	19.7 ± 1.7	27.9 ± 2.4	22.0 ± 4.5	23.1 ± 3.3	16.2 ± 1.7

4.4.4.3 *Infarct Size*

The infarct reduction afforded by postconditioning was completely abolished in the presence of the PI3K inhibitor LY294002 or Wortmannin given during the first 15 minutes of reperfusion (31.5 ± 4.1% with Postcon vs 51.7 ± 4.5% with Postcon+LY; $p<0.01$ and 56.2 ± 10.1% with Postcon+Wort; $p<0.01$) as shown in Figure 4.6. 0.02% DMSO did not influence infarct size in control groups (control 51.2 ± 3.4% vs control+DMSO 49.6 ± 9.2%). LY or Wortmannin did not influence infarct size in control groups (control+LY 56.8 ± 10.5%, control+Wortmannin 52.3 ± 13.3%).

Figure 4.6: Ischaemic Postconditioning Induced-Protection Involves the PI3K-Akt Pathway. The reduction in infarct size afforded by ischaemic postconditioning was abrogated in the presence of the PI3K inhibitors LY294002 and Wormannin. Values are mean ± SEM. * $p<0.05$.



4.5 Objective Three

To determine the role of the downstream mediators of the PI3K-Akt pathway, eNOS and p70S6 kinase, in ischaemic postconditioning-induced protection.

4.5.1 Animals

Male Sprague-Dawley rats (300 to 400g, n=30) were used for this investigation. The hearts were excised and Langendorff perfused as described in section 3.3.1 for Western blot analysis.

4.5.2 Chemicals

Wortmannin and LY294002 were made up and used as described in section 4.4.2.

4.5.3 Experimental Protocols

All hearts were subjected to 35 minutes regional ischaemia and 7 minutes reperfusion prior to Western blot analysis. The 7 minutes of reperfusion incorporates the postconditioning protocol (2 minutes) and 5 minutes of reperfusion alone. The experimental protocols used in these studies are depicted in Figure 4.7. Hearts were randomly assigned to one of the following groups:

1) **Control hearts** with 0.02% DMSO vehicle (n=5) for the first 7 minutes reperfusion;

2) **Postconditioning** (*n*=5) comprising 6 cycles of 10 seconds reperfusion followed by 10 seconds global ischaemia following the index ischaemia at the immediate onset of reperfusion;

3) **Postconditioning+LY** (n=5), hearts with the specific PI3K inhibitor LY294002 (LY, 15μmol/l) given for the first 7 minutes of reperfusion;

4) **Control+LY** (n=5), control hearts with LY given for the first 7 minutes of reperfusion;

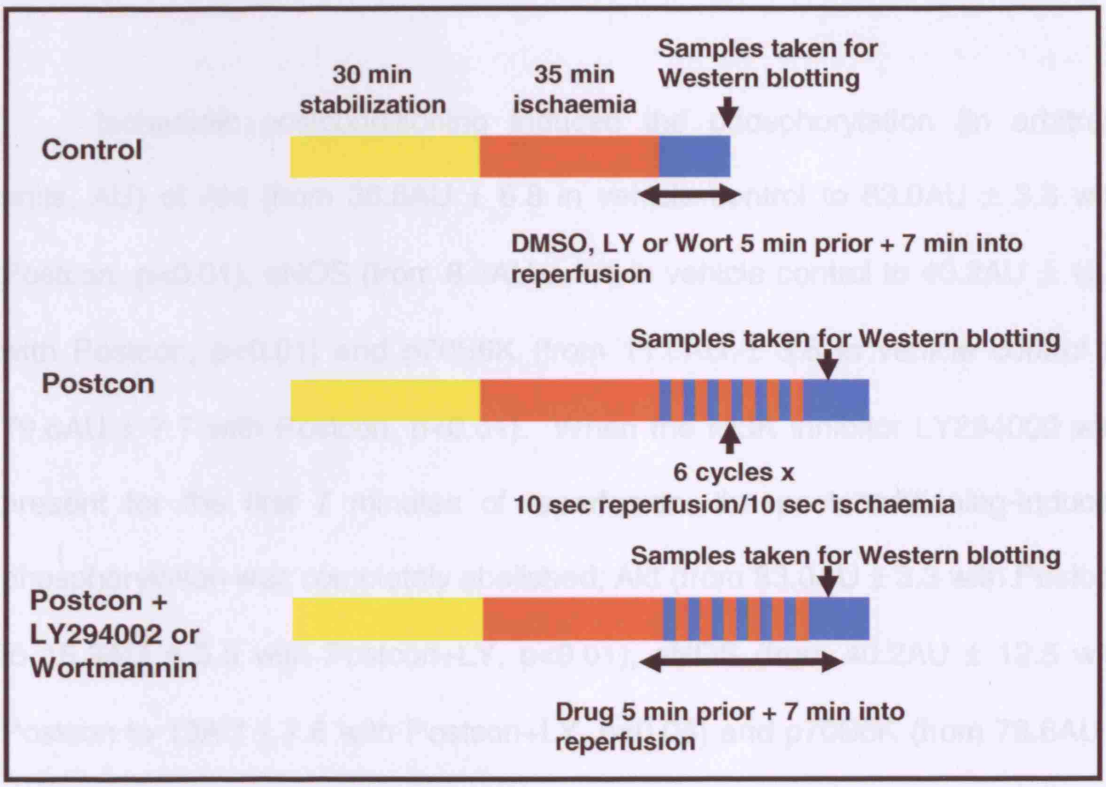
5) **Postconditioning+Wort** (n=5) with the PI3K inhibitor Wortmannin (100nmol/l) given for the first 7 minutes of reperfusion;

6) **Control+Wort** (n=5), hearts with Wortmannin given for the first 7 minutes of reperfusion.

4.5.4 Measurement of Akt, eNOS and p70S6K phosphorylation using Western blot analysis

Myocardial samples were collected at 7 minutes reperfusion from hearts that underwent the protocols as described in section 4.5.3. Western blot analysis was performed according to the methods described in section 3.3. The phosphorylation states of Akt (phospho-Akt, Ser 473), eNOS (phospho-eNOS, Ser 1177), p70S6 kinase (phospho-p70S6k, Thr 389), and total levels of Akt, eNOS and p70S6K proteins were analyzed, using antibodies obtained from Cell Signalling Technology (New England Biolabs). Levels of phosphorylated proteins were normalized to their total protein levels.

Figure 4.7: Experimental Protocols for Investigating the Role of Akt, eNOS and p70S6K in Ischaemic Postconditioning. DMSO - dimethylsulphoxide, LY - LY294002, Wort - Wortmannin.



4.5.5 Results

4.5.4.1 Exclusions

A total of 30 animals were used for this part of the study of which none were excluded.

4.5.4.2 Western Blot Analysis

Ischaemic Postconditioning Activates Akt, eNOS and p70S6K at the Time of Reperfusion

Ischaemic postconditioning induced the phosphorylation (in arbitrary units, AU) of Akt (from 36.6AU ± 6.8 in vehicle control to 83.0AU ± 3.3 with Postcon, $p<0.01$), eNOS (from 8.9AU ± 2.1 in vehicle control to 40.2AU ± 12.5 with Postcon, $p<0.01$) and p70S6K (from 11.0AU ± 3.6 in vehicle control to 79.6AU ± 7.7 with Postcon, $p<0.01$). When the PI3K inhibitor LY294002 was present for the first 7 minutes of reperfusion, the postconditioning-induced phosphorylation was completely abolished; Akt (from 83.0AU ± 3.3 with Postcon to 15.3AU ± 3.5 with Postcon+LY, $p<0.01$), eNOS (from 40.2AU ± 12.5 with Postcon to 13AU ± 7.6 with Postcon+LY, $p<0.05$) and p70S6K (from 79.6AU ± 7.7 with Postcon to 3.8AU ± 2.1 with Postcon+LY, $p<0.01$). When the PI3K inhibitor Wortmannin was present for the first 7 minutes of reperfusion, the postconditioning-induced phosphorylation of Akt and eNOS was completely blocked, and p70S6K partially blocked; Akt (from 83.0AU ± 3.3 with Postcon to 38.2AU ± 3.8 with Postcon+Wort, $p<0.01$), eNOS (from 40.2AU ± 12.5 with Postcon to 14AU ± 5.4 with Postcon+Wort, $p<0.05$) and p70S6K (from 79.6AU ± 7.7 with Postcon to 46.1AU ± 7 with Postcon+Wort, $p<0.01$) as shown in Figures 4.8 - 4.10.

The presence of LY in control hearts reduced the phosphorylation of Akt below baseline (from 36.6AU ± 6.8 in control to 19.4AU ± 4.0 in cont+LY,

$p<0.05$), had no influence on eNOS phosphorylation (from 8.9AU ± 2.1 in control to 18.0AU ± 6.0 in cont+LY, p=NS), and had no influence on p70S6K phosphorylation (from 11.0AU ± 3.6 in control to 3.3AU ± 1.9 in cont+LY, p=NS).

The presence of Wort in control hearts had no influence on Akt or eNOS phosphorylation but increased the phosphorylation of p70S6K from baseline; Akt (from 36.6AU ± 6.8 in control to 32.8AU ± 7.6 in cont+Wort, p=NS), eNOS (from 8.9AU ± 2.1 in control to 11.9AU ± 3.2 in cont+Wort, p=NS), and p70S6K (from 11.0AU ± 3.6 in control to 34.5AU ± 7.8 in cont+Wort, $p<0.01$).

Figure 4.8: Western Blots of Ischaemic Postconditioning-Induced Akt Phosphorylation. Representative Western blots demonstrating relative densitometries of phosphorylated and total levels of Akt showing that inhibiting PI3K using LY294002 or Wortmannin abolishes the phosphorylation of Akt induced by ischaemic postconditioning (*p<0.01, **p<0.05 compared with control). Cont - control, Postcon - postconditioning

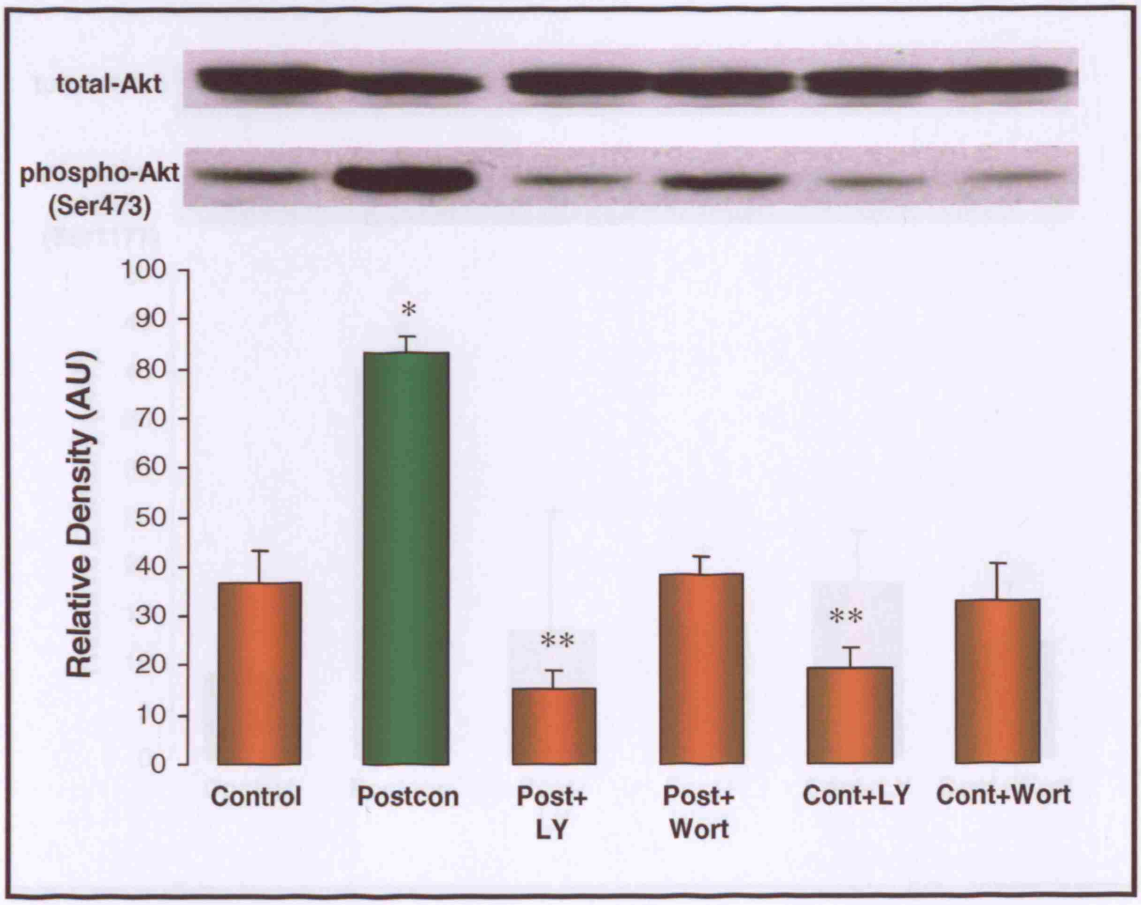


Figure 4.9: Western Blots of Ischaemic Postconditioning-Induced eNOS Phosphorylation. Representative Western blots demonstrating relative densitometries of phosphorylated and total levels of eNOS showing that inhibiting PI3K using LY294002 or Wortmannin abolishes the phosphorylation of eNOS induced by ischaemic postconditioning (*p<0.01 compared with control). Cont - control, Postcon - postconditioning

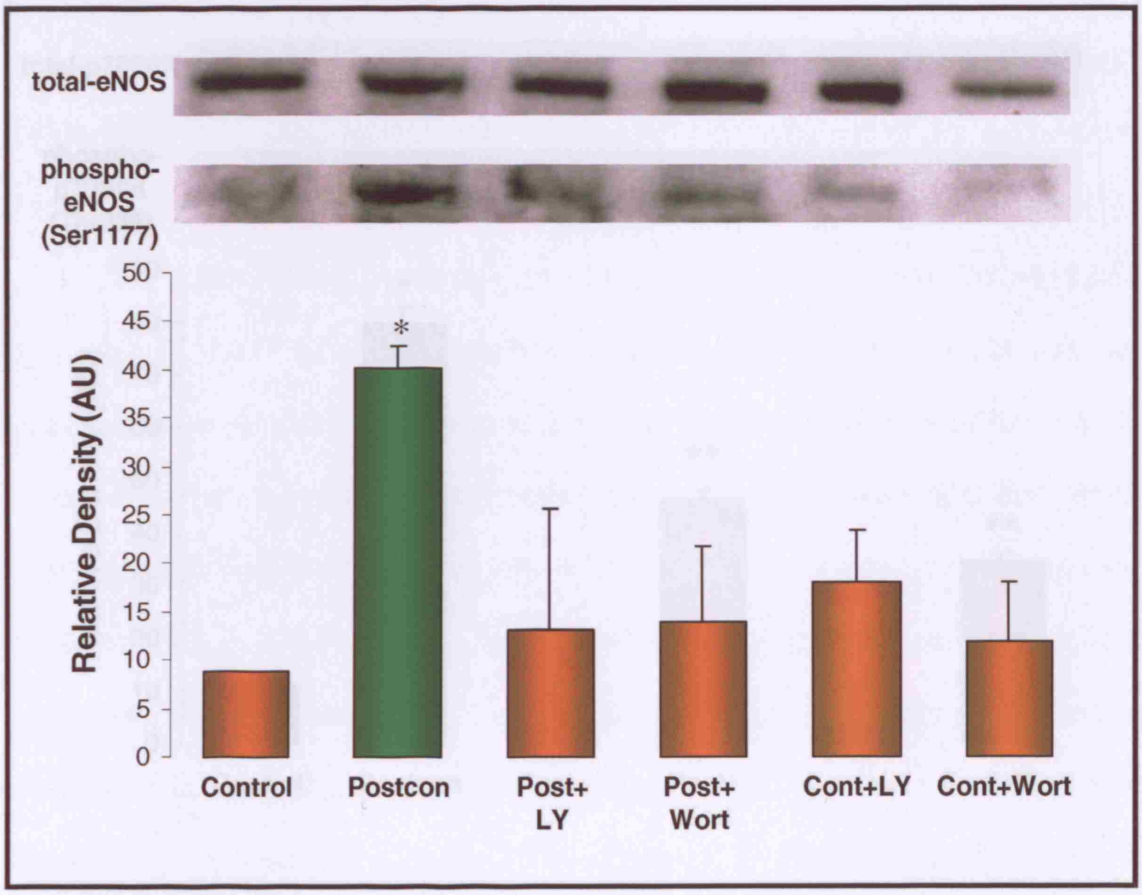
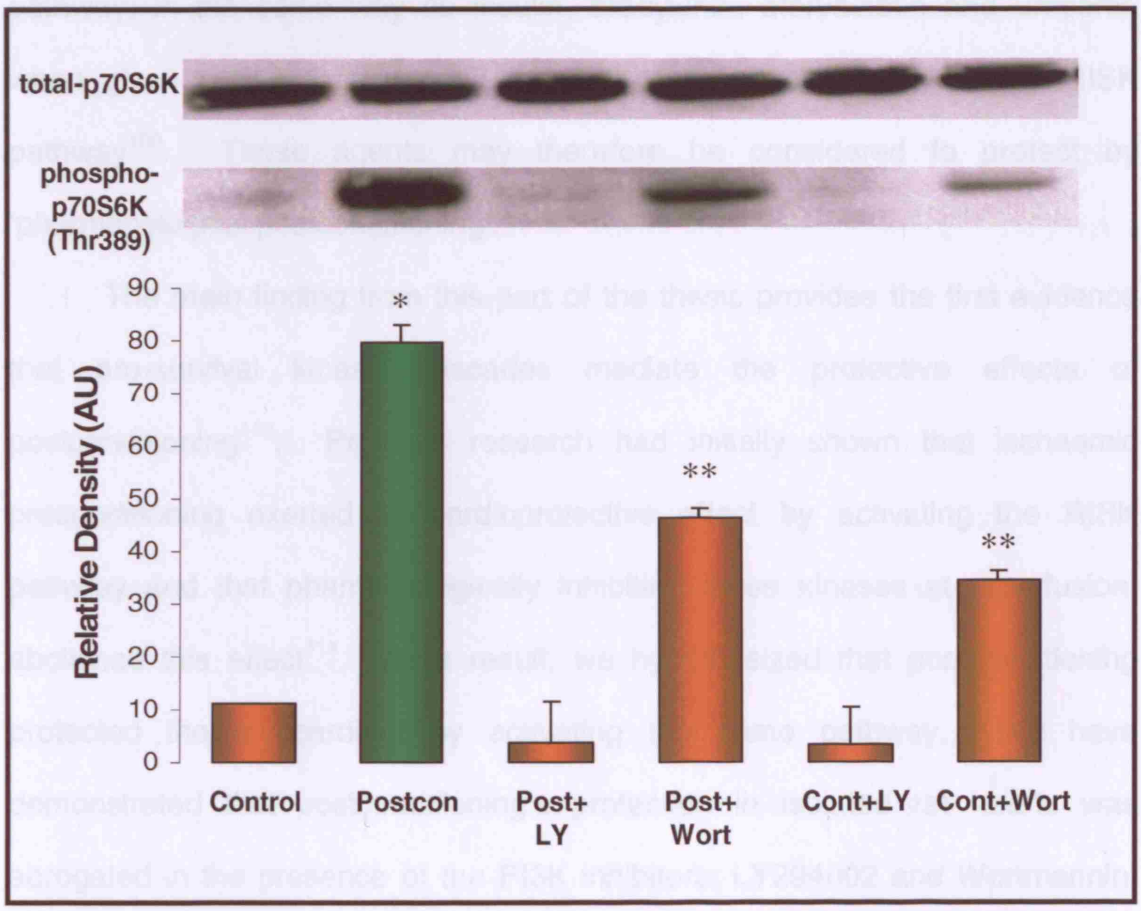


Figure 4.10: Western Blots of Ischaemic Postconditioning-Induced p70S6K Phosphorylation. Representative Western blots demonstrating relative densitometries of phosphorylated and total levels of p70S6K showing that inhibiting PI3K using LY294002 or Wortmannin abolishes the phosphorylation of p70S6K induced by ischaemic postconditioning (*p<0.01, **p<0.01 compared with control). Cont - control, Postcon - postconditioning



4.5.6 Discussion

The main finding from this part of the thesis demonstrates for the first time in the isolated perfused rat heart, that ischaemic postconditioning protects the heart against ischaemia-reperfusion injury by activating the pro-survival kinase PI3-Akt and its downstream targets, eNOS and p70S6K[112]. This data suggests that postconditioning may protect the heart by recruiting the RISK pathway, in the same way as insulin, bradykinin, atorvastatin and urocortin when given during the first few minutes of reperfusion, activate the RISK pathway[100]. These agents may therefore be considered to protect by 'pharmacological postconditioning'.

The main finding from this part of the thesis provides the first evidence that pro-survival kinase cascades mediate the protective effects of postconditioning[112]. Previous research had initially shown that ischaemic preconditioning exerted its cardioprotective effect by activating the RISK pathway and that pharmacologically inhibiting these kinases at reperfusion, abolished this effect[111]. As a result, we hypothesized that postconditioning protected the myocardium by activating the same pathway. We have demonstrated that postconditioning's protection in isolated rat hearts was abrogated in the presence of the PI3K inhibitors, LY294002 and Wortmannin, and the protection was commensurate with the phosphorylation of Akt and its downstream targets, namely p70S6K and eNOS in accordance with the RISK pathway (see Figure 1.3). Shortly afterwards, Downey and collegues[113] demonstrated that the protection afforded by postconditioning in rabbits was attenuated in the presence of PD98059 (a MEK1/2-ERK inhibitor), 5-

hydroxydecanoate (a mitochondrial K_{ATP} channel blocker), glibenclimide (non-selective K_{ATP} blocker) and L-NAME (inhibitor of nitric oxide synthase), suggesting the involvement of another component of the RISK pathway, namely the MEK1/2-ERK cascade, and downstream effectors such as production of nitric oxide and mitochondrial K_{ATP} channel opening. However, in their study it was not actually demonstrated that postconditioning led to the actual phosphorylation of ERK or eNOS. Further support for the role of ERK in postconditioning-induced cardioprotection in rabbits came from subsequent data from Przyklenk's group[133]. They too demonstrated that postconditioning-induced cardioprotection was blocked by the inhibitor PD98059 in isolated perfused rabbit hearts but in addition, postconditioning resulted in elevated phosphorylation of ERK rather than Akt. Interestingly in Downey's study[113], as seen in Kin's data[114], 4 or 6 cycles of 30 seconds reperfusion/re-occlusion produced similar reductions in infarct size. Furthermore, when the index ischaemia was extended from 30 minutes to 45 minutes, the combined effects of 1 cycle of preconditioning (5 minutes ischaemia/10 minutes reperfusion) plus 4 cycles of 30 second postconditioning resulted in an additive effect greater than either intervention alone. However, protocols using 30 minutes of index ischaemia in rats from our data[112], and 60 minutes index ischaemia in dogs in a study by Halkos et al[134], did not support this finding when pre- and postconditioning were combined. This may be explained by the different experimental protocols in these studies, and that each intervention has a maximal protective effect that can only be exposed when the index ischaemia is prolonged.

Further studies support the finding for the role of PI3 kinase in the protection afforded by postconditioning. In this regard data from Downey's group[120;122] found that postconditioning's protective effect was abolished when Wortmannin (the PI3 kinase inhibitor) was infused just before the onset of reperfusion in their rabbit model.

Interestingly, in our study the combined protective effects of IPC and postconditioning do not appear to be additive, which is in disagreement with the findings of Downey's group in which combined IPC and postconditioning in the in-vivo rabbit heart induced additive protection[113]. This discrepancy may be due to the different models of ischaemia-reperfusion injury used.

Ischaemic postconditioning as originally described in the in-vivo dog model[110] has been observed in the in-vivo rabbit[113] and rat heart[114], and in rat cardiomyocytes[115;116]. The first study investigating this phenomenon implicated a reduction in neutrophil accumulation and an improvement in endothelial function as possible mechanisms of protection[110]. Subsequent studies, including this study, have demonstrated that protection can occur in the absence of blood constituents suggesting that postconditioning may exert a direct effect on the myocyte[114].

In this regard, Vinten-Johansen's group[114] demonstrated, using the in-vivo rat heart, that postconditioning attenuated the production of reactive oxygen species (ROS) immediately at reperfusion and that ischaemic postconditioning's protection was lost if instituted after 1 minute of reperfusion. The same group have demonstrated that "hypoxic postconditioning" of neonatal rat myocytes resulted in less necrotic cell death, attenuated ROS production and reduced mitochondrial calcium loading[115].

Opening of the mitochondrial permeability transition pore (mPTP) during the first few minutes of reperfusion has been demonstrated to mediate cell death[124] and inhibiting its opening is cardioprotective[85]. Evidence for mPTP involvement in postconditioning-induced protection has been strengthened by important studies from Ovize's lab[126;127]. Using isolated rat mitochondria, this group found that postconditioning reduced mitochondrial susceptibility to calcium overload compared to controls, an effect comparable with preconditioning and NIM811 (a cyclosporin analogue specific mPTP inhibitor). Subsequent work from the same lab suggests that this mPTP opening is PI3K regulated, as evidenced by the abrogation of mitochondrial resistance to calcium overload in the presence of Wortmannin or LY294002. Data from Serviddio and collegues[135] suggests that postconditioning induced protection correlates with reduced mitochondrial production of reactive oxygen species during early reperfusion. However, the exact role of the mPTP in postconditioning is still unclear, and further studies in this area are needed.

The term 'ischaemic postconditioning' is probably a misnomer as the word 'conditioning' implies that the process prepares the myocardium for the ischaemic event, as perceived in ischaemic preconditioning. Ischaemic postconditioning may simply constitute a variation of controlled reperfusion, which previous studies have demonstrated to be cardioprotective[96]. Therefore ischaemic postconditioning may be considered a 'passive process' which modifies reperfusion injury rendering the cell and mitochondria more resistant to the biochemical and metabolic perturbations which occurs in the transition from ischaemia to reperfusion. We show that ischaemic postconditioning may also

be an 'active process' by activating pro-survival kinases such as the PI3K-Akt pathway in accordance with the RISK pathway (see Figure 1.3).

In conclusion, our study demonstrates that ischaemic postconditioning significantly reduces infarct size in isolated perfused rat hearts and that the effects of IPC and postconditioning combined are not additive. In addition we show for the first time that protection is mediated via the PI3K-Akt pro-survival signalling cascade and its downstream targets, namely, eNOS and p70S6K. Interventions such as ischaemic postconditioning, which target the first few minutes of reperfusion, may offer greater opportunity for protection clinically such as at the time of thrombolysis, angioplasty and cardiac surgery.

To date, ischaemic postconditioning has only been demonstrated to be an effective myocardial protective strategy in normal disease-free hearts. However, in the human population many of those at risk from ischaemic heart disease commonly have other risk factors such as hypertension and diabetes. In the next section of this thesis we will examine the effects of ischaemic postconditioning and the more commonly known protective phenomenon of IPC in type 2 diabetic hearts.

Chapter Five: CARDIOPROTECTION IN TYPE 2 DIABETES

5.1 Introduction

Diabetes mellitus is a major risk factor for ischaemic heart disease, the latter of which, is the leading cause of death in the western world[136] and accounts for more than 50% of deaths in the diabetic population[137]. Following a myocardial ischaemic event, diabetes is associated with increased adverse outcomes in terms of both morbidity and mortality over the short and long term[138-141]. In patients with diabetes, the mortality rate following an acute myocardial infarction or coronary bypass surgery is almost double that compared with non-diabetics[142;143]. Therefore, reducing the consequences of coronary artery disease using strategies which target ischaemia-reperfusion injury would be particularly beneficial in this population.

The new phenomenon of ischaemic postconditioning has so far only been observed in disease-free animal hearts however, as already seen, those most likely to benefit from this protective strategy are patients with diseased hearts from specific coronary artery disease related risk factors.

Unfortunately, the current literature regarding whether the diabetic myocardium can be protected by the phenomenon of ischaemic preconditioning is conflicting[144]. Whilst some studies have reported that the diabetic heart can be protected using ischaemic preconditioning[145-147], the majority have reported no protective effect[148-154], although almost all of these studies have used a type

1 diabetic model, the least common form of human diabetes. For example, Liu et al[145] demonstrated that IPC achieved using 3 cycles of 5 minutes ischaemia and 5 minutes reperfusion, reduced infarct size in open chest rats by 80% in their streptozotocin-induced diabetic model. However, Kersten et al did not find any reduction in infarct size from IPC using 4 cycles of 5 minutes ischaemia and 5 minutes reperfusion in alloxan and streptozotocin-induced diabetic dogs[148], a finding supported by data from Koltai's group[150] in alloxan-induced diabetic rabbits and Flyvbjerg's group using type 2 diabetic Goto-Kakizaki rats[149].

The importance of the PI3 kinase-Akt pro-survival pathway in ischaemic preconditioning in the normal heart was first demonstrated by Tong and colleagues[44], and has been further supported by subsequent studies from our group[46]. However, the cellular signalling pathways that specifically mediate the effects of ischaemic preconditioning in the **diabetic** myocardium have not been elucidated. Recent data using type 2 diabetic models have provided clues as to the possible defects in the cell survival cascades in a variety of tissues as well as the heart that may be responsible for the conflicting results seen and the failure of protective strategies. For example, defects in the insulin receptor β, insulin receptor substrate-1 (IRS-1) and GLUT4 protein[155], protein kinase B[156], basal and insulin-stimulated Akt, ERK and PI3 kinase[157], have all been demonstrated in diabetic animal models.

In trying to identify rats in which we could examine the pathology of diabetes without using diabetes-inducing chemicals, we identified that the GK rat is a selectively in-bred model of type 2 diabetes developed from the Wistar rat and which has many similarities to the human form of the disease [158;159]. It has been used extensively as a type 2 diabetic research model [160].

Therefore, the aims of this section of the study were: 1) to determine whether the in-bred lean model of type 2 diabetes, namely the Goto-Kakizaki (GK) rat can be protected by ischaemic postconditioning, 2) if postconditioning fails to protect, to determine whether the Goto-Kakizaki rat can be protected by the more commonly known protective phenomenon of IPC, and 3) to determine whether IPC in type 2 diabetic hearts depends on intact signalling through the PI3K-Akt pathway to achieve a reduction in myocardial injury.

5.2 Hypothesis Two

Ischaemic postconditioning protects the type 2 diabetic rat heart against the effects of ischaemic-reperfusion injury.

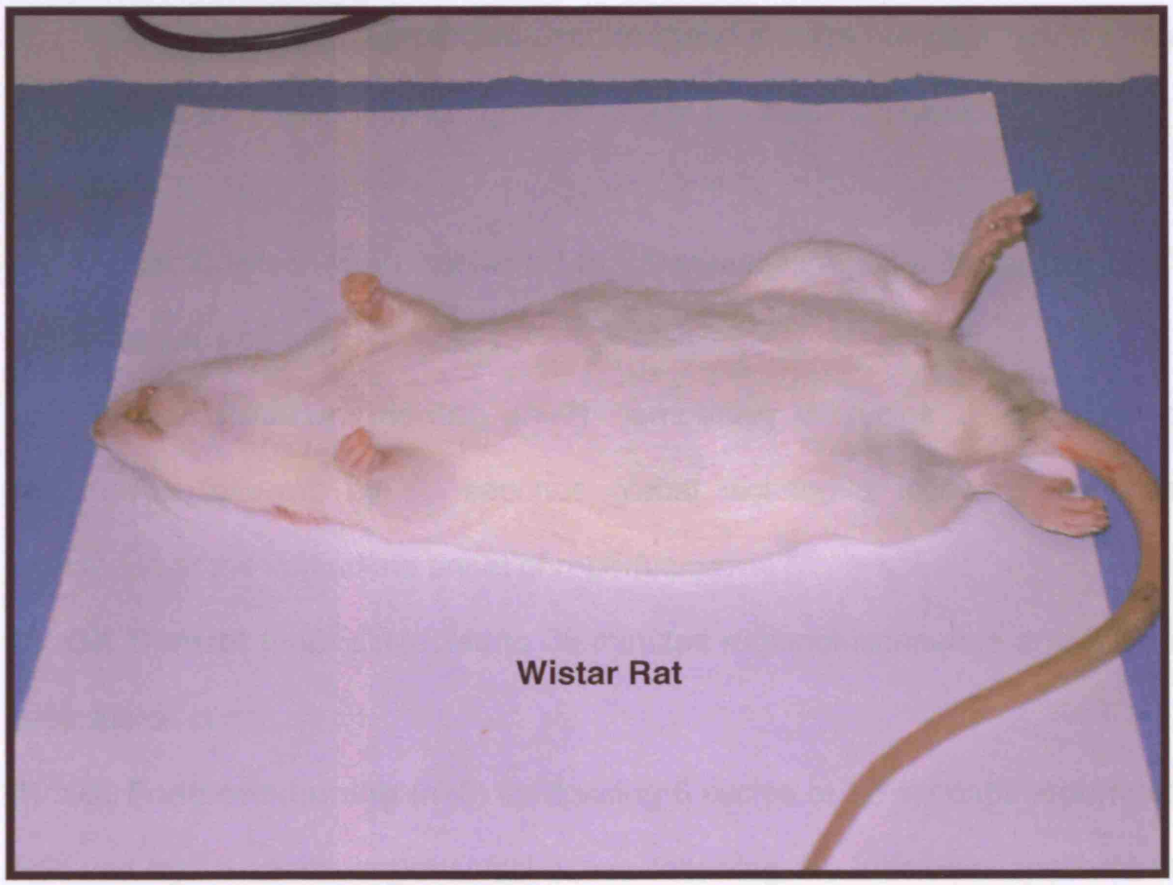
5.3 Objective One

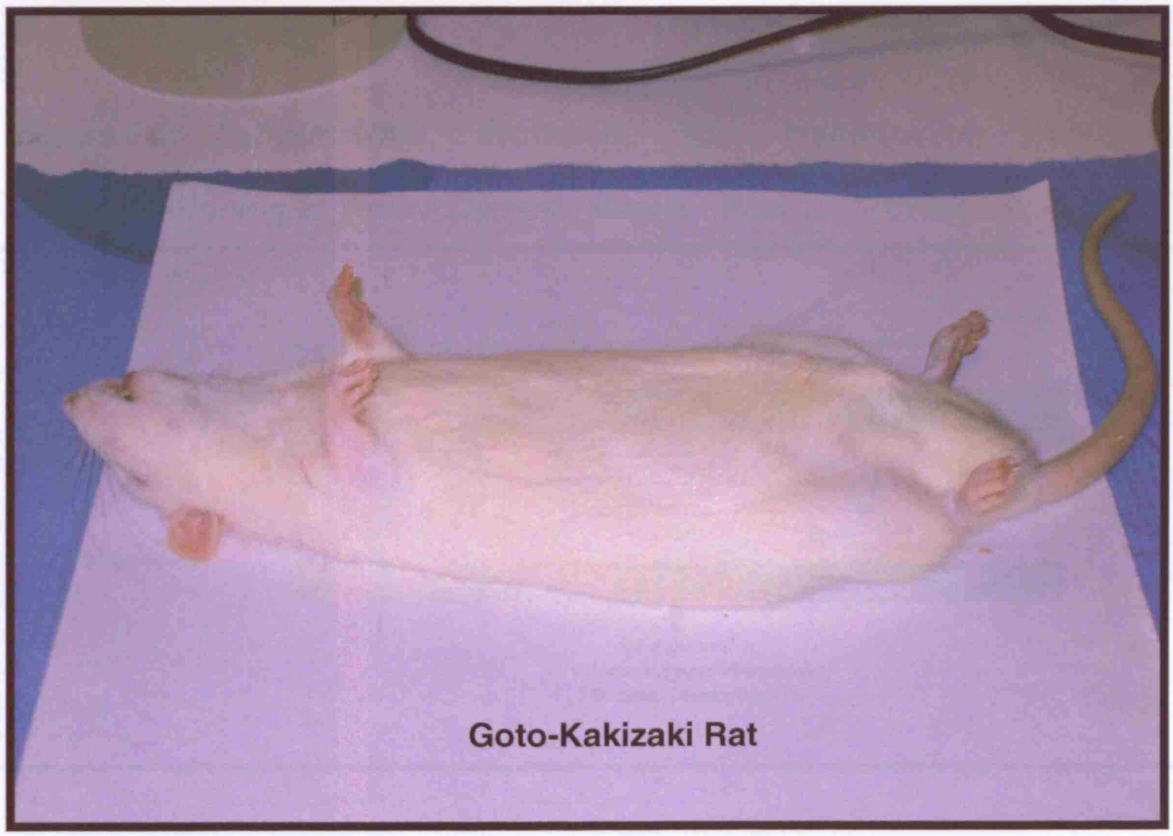
To investigate whether ischaemic postconditioning protects the type 2 diabetic rat myocardium from ischaemia-reperfusion injury.

5.3.1 Animals

Male Wistar rats (350 to 480g, n=17) and male Goto-Kakizaki rats (300 to 550g, n=19) were used for this investigation and are depicted in Figure 5.1. The hearts were excised and Langendorff perfused as described in section 3.2.1. All hearts were subjected to 35 minutes regional ischaemia and 2 hours reperfusion before infarct analysis by TTC staining.

Figure 5.1: Phenotypic Appearance of Wistar and Goto-Kakizaki Rats.





5.3.2 Experimental Protocols

The experimental protocols used in these studies are depicted in Figure 5.2. Hearts were randomly assigned to one of the following groups:

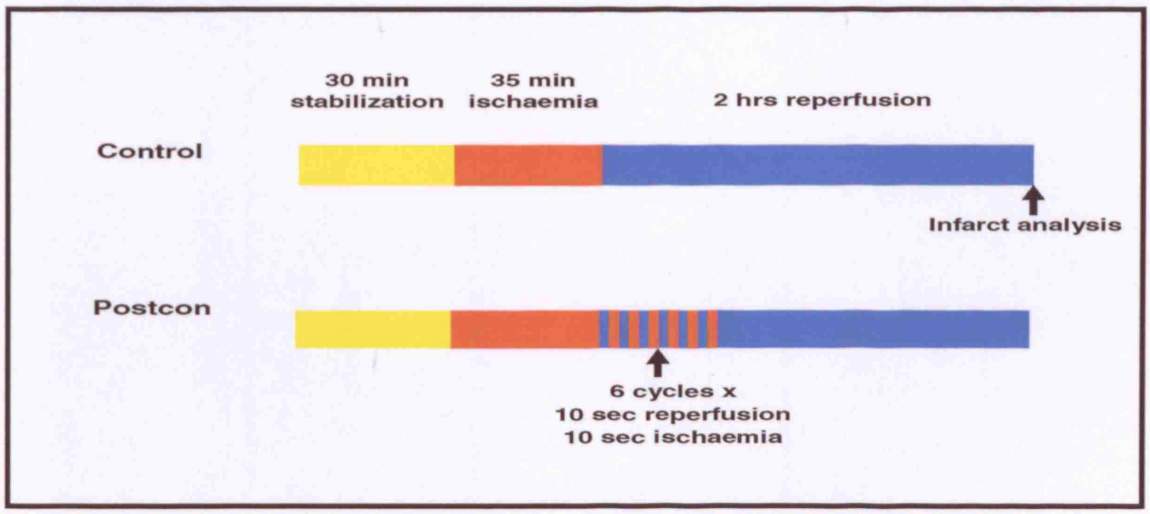
1) **Wistar Control** (n=8) subjected to 35 minutes regional ischaemia and 2 hours reperfusion only;

2) **Wistar Postconditioning** (n=7) comprising 6 cycles of 10 seconds reperfusion followed by 10 seconds global ischaemia following the index ischaemia at the immediate onset of reperfusion;

3) **GK Control** (n=8) subjected to 35 minutes regional ischaemia and 2 hours reperfusion only;

4) **GK Postconditioning** (n=8) comprising 6 cycles of 10 seconds reperfusion followed by 10 seconds global ischaemia following the index ischaemia at the immediate onset of reperfusion.

Figure 5.2: Experimental Protocols for Investigating Ischaemic Postconditioning in Type 2 Diabetic Hearts. Postcon - postconditioning.



5.3.3 Results

5.3.3.1 Exclusions

A total of 36 animals were used for this part of the study of which 5 were excluded due to the criteria as described in section 3.2.1.4.

5.3.3.2 Cardiac Functional Data

Body weights and AAR (area at risk) between experimental groups are shown in Table 5.1. At the end of the stabilization period, coronary flow and RPP were comparable between all groups with no significant differences. Similarly, coronary flow and RPP (rate pressure product) were reduced after the induction of ischaemia to a similar degree and recovered to the same extent after the onset of reperfusion in all groups (see Figure 5.3 A+B, Table 5.2 and 5.3).

Table 5.1: Animal Characteristics of Experimental Groups. Values are mean ± SEM. *p<0.05. Postcon - Postconditioning, GK - Goto Kakizaki

Group	Number	Body Weight (g)	Risk Volume (cm^3)
Wistar Control	8	484.8 ± 17.1*	0.582 ± 0.035
Wistar Postcon	7	355.6 ± 10.9	0.490 ± 0.029
GK Control	8	370.6 ± 8.9	0.561 ± 0.042
GK Postcon	8	359.1 ± 4.8	0.519 ± 0.029

Figure 5.3: Cardiac Functional Data. Coronary flow rate and RPP changes throughout the ischaemic-reperfusion protocol. Points represent mean values, standard error bars have been removed for clarity. S – stabilization, I – ischaemia, R – reperfusion, Wist - Wistar, GK - Goto Kakizaki, Ct - Control, Post - Postconditioning

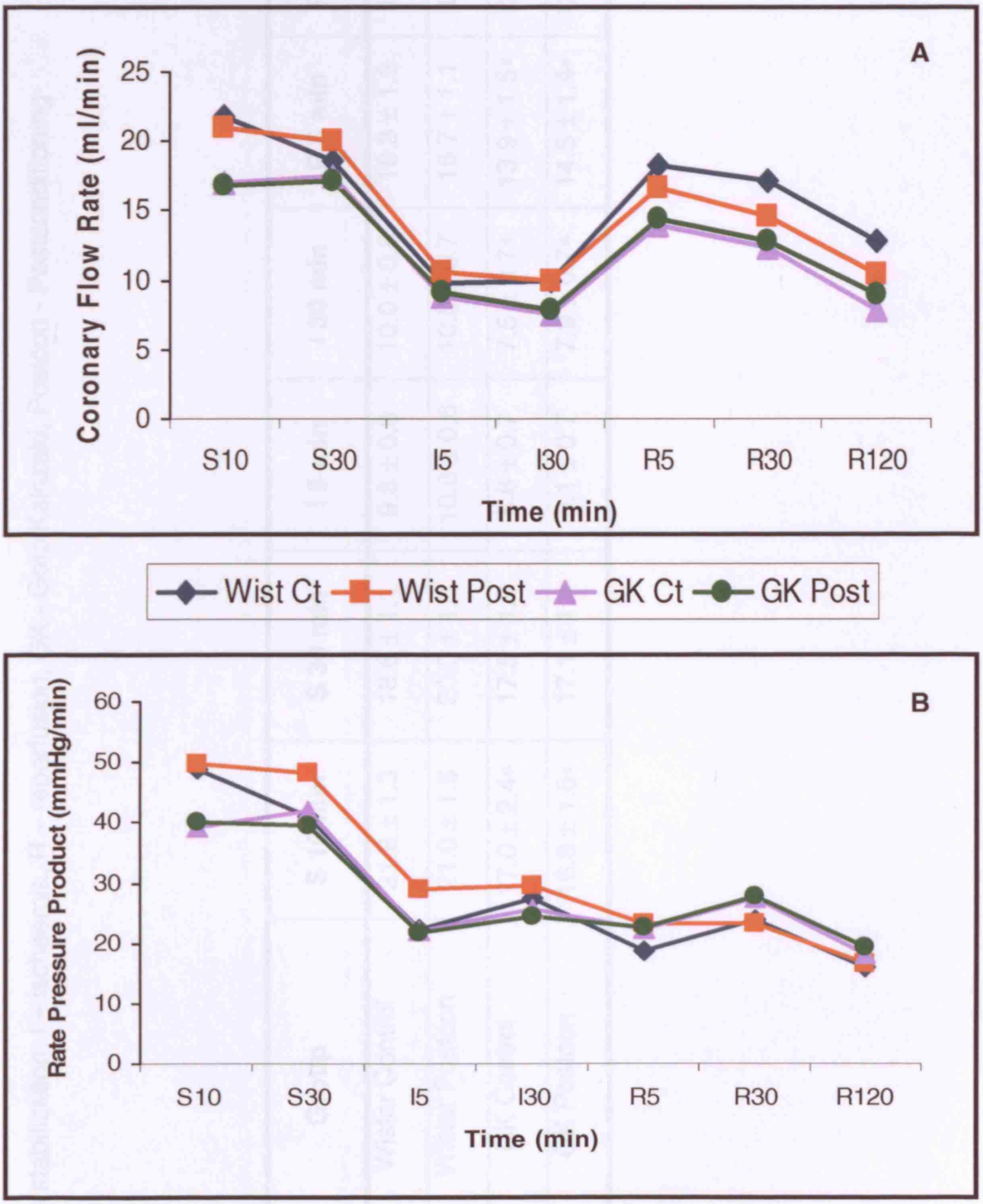


Table 5.2: Coronary Flow Rate in Experimental Groups (ml/min). Values are mean ± SEM. * p <0.05 compared with control.

S – stabilization, I – ischaemia, R – reperfusion, GK - Goto Kakizaki, Postcon - Postconditioning

Group	S 10 min	S 30 min	I 5 min	I 30 min	R 5 min	R 30 min	R 120 min
Wistar Control	21.8 ± 1.3	18.6 ± 1.1	9.8 ± 0.8	10.0 ± 0.8	18.3 ± 1.9	17.1 ± 1.9	12.9 ± 1.8
Wistar Postcon	21.0 ± 1.6	20.0 ± 1.6	10.6 ± 0.6	10.0 ± 0.7	16.7 ± 1.1	14.6 ± 0.9	10.4 ± 0.6
GK Control	17.0 ± 2.4*	17.5 ± 1.6	8.8 ± 0.7	7.5 ± 0.7*	13.9 ± 1.5*	12.3 ± 1.4*	7.9 ± 0.8*
GK Postcon	16.8 ± 1.6*	17.1 ± 1.4	9.1 ± 0.7	7.9 ± 0.7*	14.5 ± 1.4*	12.8 ± 1.4*	9.0 ± 0.6*

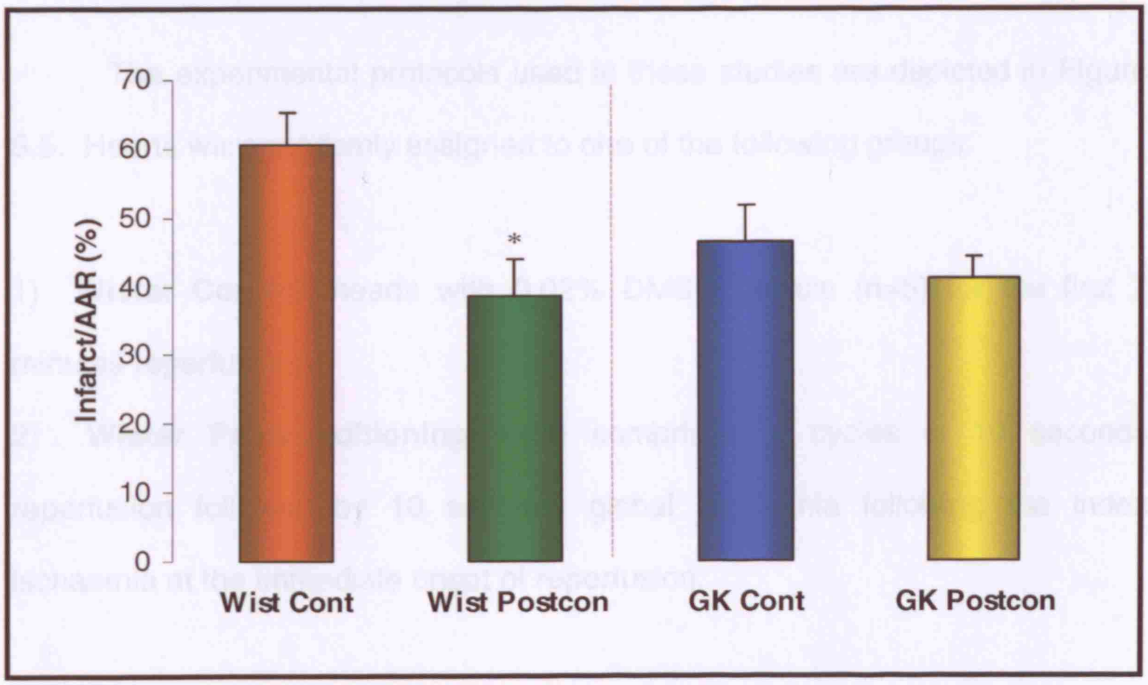
Table 5.3: Rate Pressure Product in Experimental Groups (mm Hg/min x 10^3). Values are mean ± SEM. * p <0.05 compared with control. S – stabilization, I – ischaemia, R – reperfusion, GK - Goto Kakizaki, Postcon - Postconditioning

Group	S 10 min	S 30 min	I 5 min	I 30 min	R 5 min	R 30 min	R 120 min
Wistar Control	48.7 ± 4.2	41.1 ± 3.3	22.4 ± 2.5	27.5 ± 2.2	18.8 ± 2.8	23.9 ± 2.9	16.1 ± 3.3
Wistar Postcon	49.7 ± 2.0	48.1 ± 1.8	28.8 ± 1.8*	29.6 ± 2.5	23.1 ±1.2	23.2 ± 2.1	16.7 ± 1.6
GK Control	39.3 ± 4.2	41.9 ± 5.0	21.9 ± 1.8	25.5 ± 2.5	22.2 ± 2.4	27.4 ± 3.0	18.0 ± 2.4
GK Postcon	40.1 ± 4.8	39.4 ± 4.5	21.8 ± 2.7	24.3 ± 3.7	22.6 ± 2.1	27.7 ± 2.6	19.2 ± 1.5

5.3.3.3 Infarct Size

Infarct size, represented as a percentage of the area at risk (AAR), was significantly reduced in the Wistar Postconditioning group compared with Wistar control hearts (Wistar Control 60.7 ± 4.5% vs Wistar Postcon 38.7 ± 5.4% $p<0.01$). However, ischaemic postconditioning in the diabetic GK rats did not reduce infarct size when compared with GK control hearts (GK Control 46.6 ± 5.2% vs GK Postcon 41.0 ± 3.2%, p=NS) as shown in Figure 5.4. Interestingly, GK control hearts exhibited smaller infarcts compared with Wistar control hearts (GK Control 46.6 ± 5.2% vs Wistar Control 60.7 ± 4.5%, $p<0.05$).

Figure 5.4: Ischaemic Postconditioning Fails to Protect the Isolated Diabetic Heart from Ischaemic-Reperfusion Injury. The reduction in infarct size afforded by ischaemic postconditioning observed in normal Wistar hearts was not seen in diabetic GK hearts. Values are mean ± SEM. * $p<0.05$.



5.4 Objective Two

To determine the role of the pro-survival PI3K-Akt pathway in ischaemic postconditioning in type 2 diabetes.

5.4.1 Animals

Male Wistar rats (300 to 400g, n=10) and male Goto-Kakizaki rats (300 to 400g, n=10) were used for this part of the investigation. The hearts were excised and Langendorff perfused as described in section 3.2.1. All hearts were subjected to 35 minutes regional ischaemia and 7 minutes reperfusion prior to Western blot analysis. The 7 minutes of reperfusion incorporates the postconditioning protocol (2 minutes) and 5 minutes of reperfusion alone.

5.4.2 Experimental Protocols

The experimental protocols used in these studies are depicted in Figure 5.5. Hearts were randomly assigned to one of the following groups:

1) **Wistar Control**, hearts with 0.02% DMSO vehicle (n=5) for the first 7 minutes reperfusion;

2) **Wistar Postconditioning** (n=5) comprising 6 cycles of 10 seconds reperfusion followed by 10 seconds global ischaemia following the index ischaemia at the immediate onset of reperfusion;

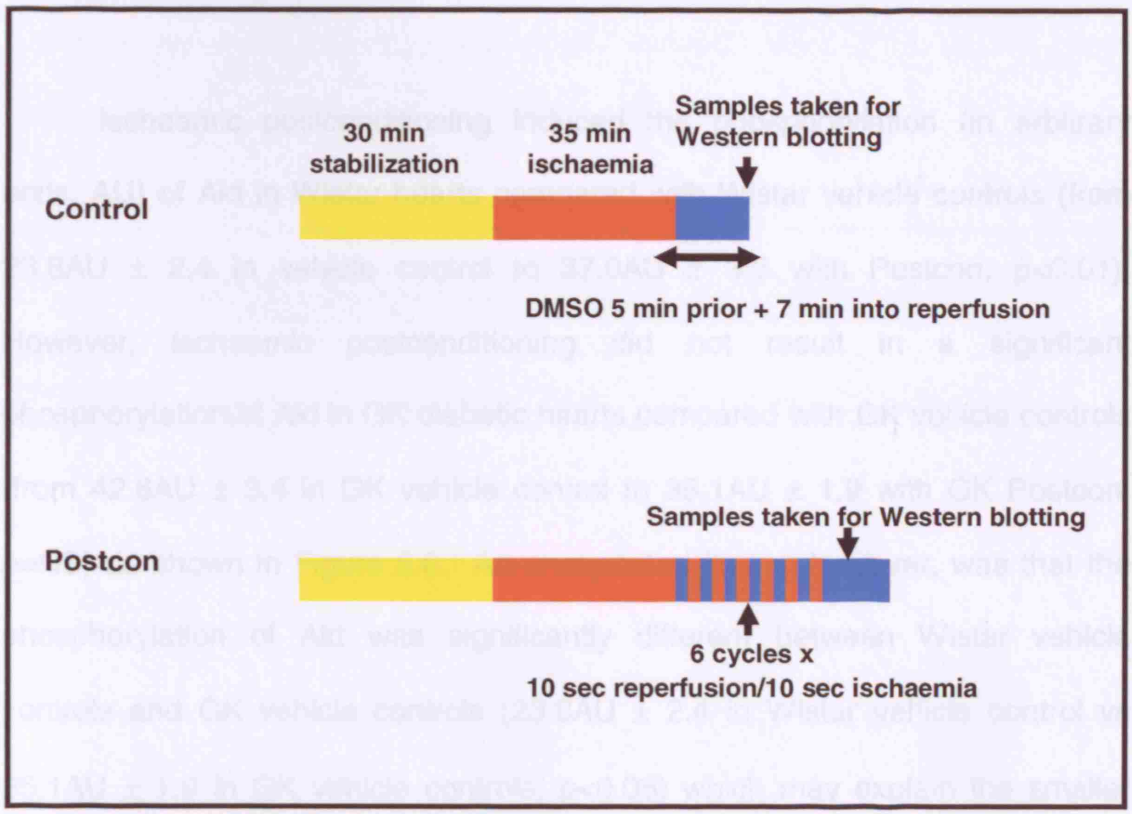
3) **GK Control**, hearts with 0.02% DMSO vehicle (n=5) for the first 7 minutes reperfusion;

4) **GK Postconditioning** (n=5) comprising 6 cycles of 10 seconds reperfusion followed by 10 seconds global ischaemia following the index ischaemia at the immediate onset of reperfusion.

5.4.3 Measurement of Akt Phosphorylation Using Western Blot Analysis

Myocardial samples were collected at 7 minutes reperfusion from hearts that underwent the protocols as described in section 5.3.5. Western blot analysis was performed according to the methods described in section 3.3. The phosphorylation state of Akt (phospho-Akt, Ser 473) and total levels of Akt protein were analyzed, using antibodies obtained from Cell Signalling Technology (New England Biolabs). Levels of phosphorylated proteins were normalized to their total protein levels.

Figure 5.5: Experimental Protocols for Investigating the Role of Akt in Ischaemic Postconditioning in Type 2 Diabetic Hearts. Postcon - postconditioning, DMSO - dimethylsulphoxide



5.4.5 Results

5.4.5.1 Exclusions

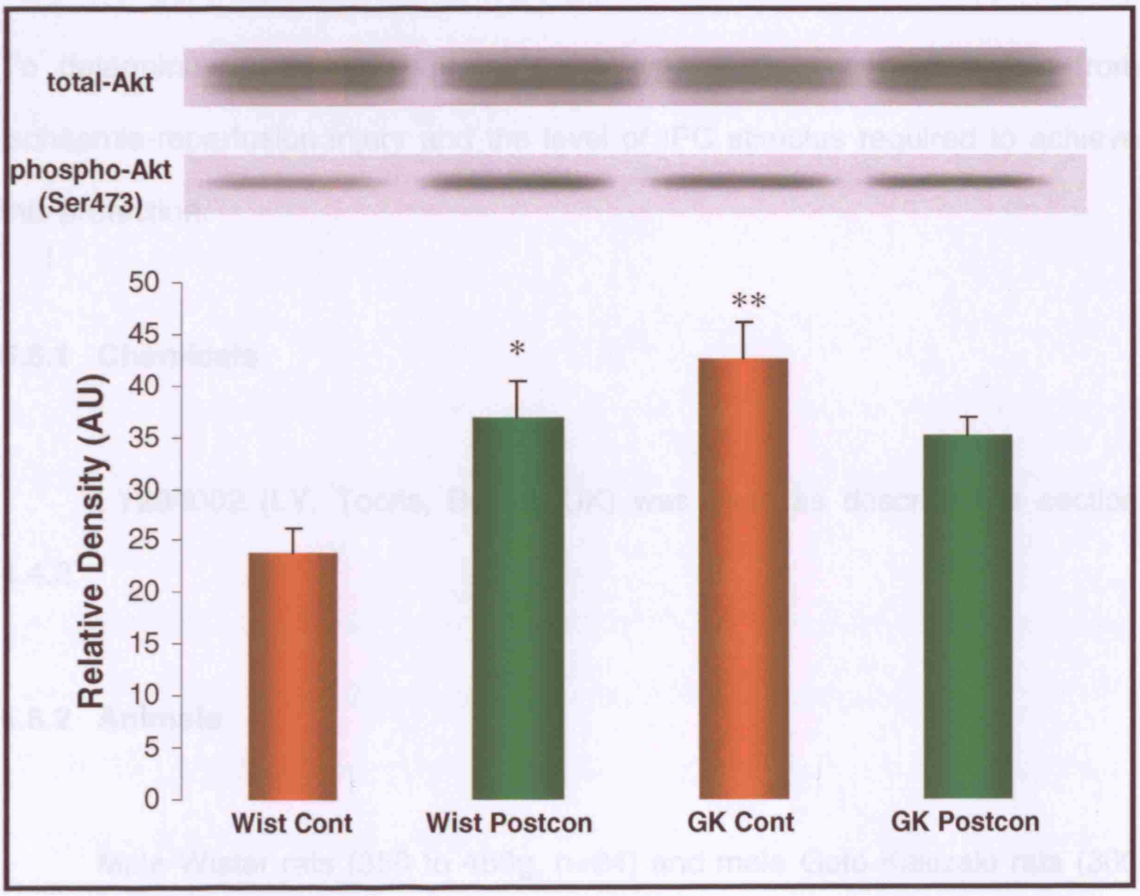
A total of 20 animals were used for this part of the study of which none were excluded.

5.4.5.2 Western Blot Analysis

Ischaemic Postconditioning Fails to Activate Akt at the Time of Reperfusion in the Type 2 Diabetic Heart

Ischaemic postconditioning induced the phosphorylation (in arbitrary units, AU) of Akt in Wistar hearts compared with Wistar vehicle controls (from 23.8AU ± 2.4 in vehicle control to 37.0AU ± 3.3 with Postcon, $p<0.01$). However, ischaemic postconditioning did not result in a significant phosphorylation of Akt in GK diabetic hearts compared with GK vehicle controls (from 42.6AU ± 3.4 in GK vehicle control to 35.1AU ± 1.9 with GK Postcon, p=NS) as shown in Figure 5.6. An unexpected finding however, was that the phosphorylation of Akt was significantly different between Wistar vehicle controls and GK vehicle controls (23.8AU ± 2.4 in Wistar vehicle control vs 35.1AU ± 1.9 in GK vehicle controls, $p<0.05$) which may explain the smaller infarct size seen in GK controls described in section 5.3.3.3.

Figure 5.6: Western Blots of Ischaemic Postconditioning in Type 2 Diabetic Hearts. Representative Western blots demonstrating relative densitometries of phosphorylated and total levels of Akt showing that ischaemic postconditioning fails to induce phosphorylation of Akt above GK controls (*p<0.01, **p<0.05 compared with Wistar control). Wist - Wistar, GK - Goto Kakizaki, Cont - Control, Postcon - Postconditioning.



5.5 Hypothesis Three

Ischaemic preconditioning (IPC) can protect the type 2 diabetic rat heart against the effects of ischaemic-reperfusion injury.

5.6 Objective One

To determine whether IPC protects the type 2 diabetic myocardium from ischaemia-reperfusion injury and the level of IPC stimulus required to achieve this protection.

5.6.1 Chemicals

LY294002 (LY, Tocris, Bristol, UK) was used as described in section 4.4.2.

5.6.2 Animals

Male Wistar rats (350 to 480g, n=24) and male Goto-Kakizaki rats (300 to 550g, n=38) were used for this investigation. The hearts were excised and Langendorff perfused as described in section 3.2.1. All hearts were subjected to 35 minutes regional ischaemia and 2 hours reperfusion before infarct analysis by TTC staining.

5.6.3 Blood glucose and glycated haemoglobin (HbA1c) assessment

Samples for non-fasting blood glucose (n=92) and HbA1c (n=45) were taken immediately after excision of the heart. Blood glucose measurements (mmol/l) were determined using an ABL 700 series blood gas analyzer (Radiometer, Copenhagen) and HbA1c measurements (%) were determined by an antibody-colorimetric assay using a Cobas Mira Plus analyzer (Roche Diagnostic Systems). Infarct size - glucose - HbA1c correlations were calculated by linear regression analysis.

5.6.4 Experimental Protocols

The experimental protocols used in this part of the study are depicted in Figure 5.7. Hearts were randomly assigned to one of the following groups:

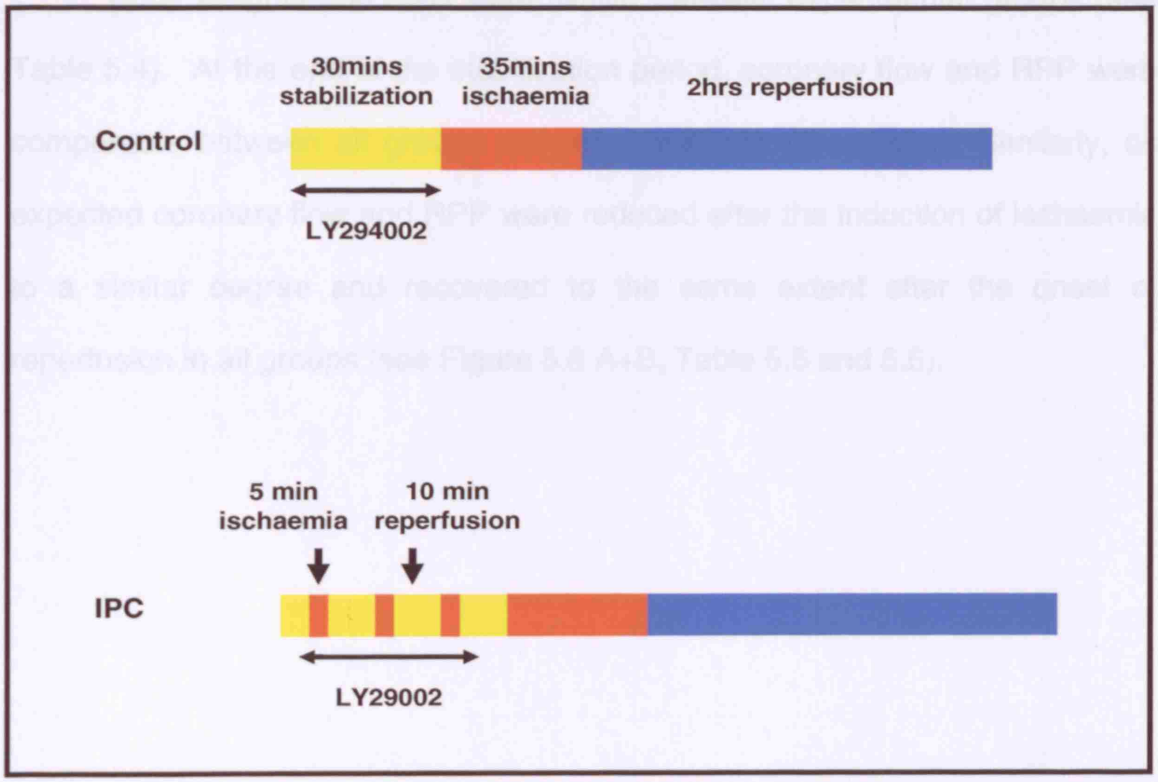
1) **Wistar IPC1** (n=8), comprising 1 cycle of IPC of 5 minutes global ischaemia followed by 10 minutes reperfusion, prior to the index ischaemia;
2) **Wistar IPC2** (n=8), comprising 2 cycles of IPC prior to the index ischaemia;
3) **Wistar IPC3** (n=8), comprising 3 cycles of IPC prior to the index ischaemia;
4) **GK IPC1** (n=9), comprising 1 cycle of IPC of 5 minutes global ischaemia followed by 10 minutes reperfusion, prior to the index ischaemia;
5) **GK IPC2** (n=9), comprising 2 cycles of IPC prior to the index ischaemia;
6) **GK IPC3** (n=8), comprising 3 cycles of IPC prior to the index ischaemia;

7) **GK IPC3+LY** (n=6), hearts were given the PI3K inhibitor LY294002 (LY, 15μmol/l) 5 minutes before and throughout the duration of the preconditioning protocol with a 5 minute washout prior to the index ischaemia;

8) **GK Control+LY** (n=6), hearts with LY given during stabilization.

Wistar and GK control hearts were the same hearts as in section 5.3.2 and have been included here for comparison.

Figure 5.7: Experimental Protocols for Investigating Ischaemic Preconditioning in Type 2 Diabetic Hearts. IPC - ischaemic preconditioning,



5.6.5 Results

5.6.5.1 Exclusions

A total of 68 animals were used for this part of the study of which 6 were excluded due to the criteria as described in section 3.2.1.4.

5.6.5.2 Cardiac Functional Data

Body weights and AAR were similar between experimental groups (see Table 5.4). At the end of the stabilization period, coronary flow and RPP were comparable between all groups with no significant differences. Similarly, as expected coronary flow and RPP were reduced after the induction of ischaemia to a similar degree and recovered to the same extent after the onset of reperfusion in all groups (see Figure 5.8 A+B, Table 5.5 and 5.6).

Table 5.4: Animal Characteristics of Experimental Groups. Values are mean ± SEM. GK - Goto Kakizaki, IPC - ischaemic preconditioning, Cont - control, LY - LY294002

Group	Number	Body Weight (g)	Risk Volume (cm^3)
Wistar IPC1	8	409.9 ± 19.6	0.626 ± 0.038
Wistar IPC2	8	437.6 ± 28.5	0.592 ± 0.053
Wistar IPC3	8	377.1 ± 7.9	0.574 ± 0.039
GK IPC1	9	376.3 ± 11.7	0.572 ± 0.028
GK IPC2	9	361.4 ± 8.7	0.530 ± 0.029
GK IPC3	8	373.0 ± 4.1	0.522 ± 0.035
GK IPC3+LY	6	371.3 ± 9.2	0.534 ± 0.027
GK Cont+LY	6	342.8 ±25.2	0.524 ± 0.055

Figure 5.8: Cardiac Functional Data. Coronary flow rate and RPP changes throughout the ischaemic-reperfusion protocol. Points represent mean values, standard error bars have been removed for clarity. S – stabilization, I – ischaemia, R – reperfusion, Ct - control

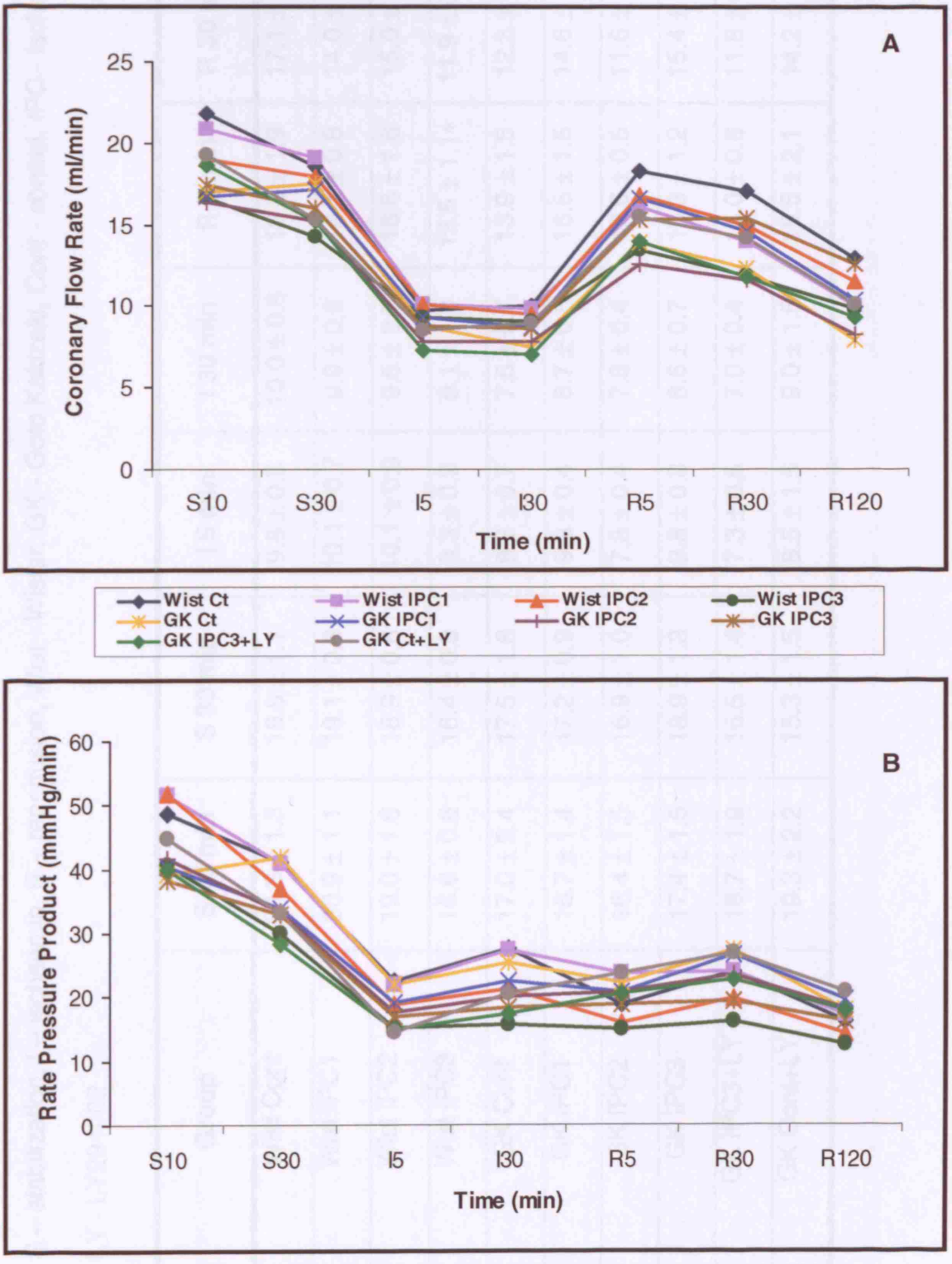


Table 5.5: Coronary Flow Rate in Experimental Groups (ml/min). Values are mean ± SEM. * p <0.05 compared with control. S – stabilization, I – ischaemia, R – reperfusion, Wist - Wistar, GK - Goto Kakizaki, Cont - control, IPC - ischaemic preconditioning, LY - LY294002

Group	S 10 min	S 30 min	I 5 min	I 30 min	R 5 min	R 30 min	R 120 min
Wist Cont	21.8 ± 1.3	18.6 ± 1.1	9.8 ± 0.8	10.0 ± 0.8	18.3 ± 1.9	17.1 ± 1.9	12.9 ± 1.8
Wist IPC1	20.9 ± 1.1	19.1 ± 0.8	10.1 ± 0.7	9.9 ± 0.6	16.1 ± 0.8	14.0 ± 0.9	10.0 ± 0.7
Wist IPC2	19.0 ± 1.8	18.9 ± 0.8	10.1 ± 0.9	9.5 ± 0.7	16.8 ± 1.6	15.0 ± 1.4	11.5 ± 1.4
Wist IPC3	16.6 ± 0.8	16.4 ± 0.9	9.3 ± 0.9	9.1 ± 1.0	13.5 ± 1.1*	11.9 ± 0.8*	9.9 ± 0.8*
GK Cont	17.0 ± 2.4	17.5 ± 1.6	8.8 ± 0.7	7.5 ± 0.7	13.9 ± 1.5	12.3 ± 1.4	7.9 ± 0.8
GK IPC1	16.7 ± 1.4	17.2 ± 0.9	9.3 ± 0.4	8.7 ± 0.5	16.6 ± 1.6	14.6 ± 1.6	10.4 ± 0.9
GK IPC2	16.4 ± 1.5	16.9 ± 1.0	7.8 ± 0.4	7.8 ± 0.4	12.6 ± 0.5	11.6 ± 0.5	8.3 ± 0.2
GK IPC3	17.4 ± 1.5	18.9 ± 1.3	8.8 ± 0.8	8.6 ± 0.7	15.3 ± 1.2	15.4 ± 1.3	12.6 ± 1.4*
GK IPC3+LY	18.7 ± 1.9	16.5 ± 1.4	7.3 ± 0.5	7.0 ± 0.4	14.0 ± 0.6	11.8 ± 0.7	9.3 ± 0.4
GK Cont+LY	19.3 ± 2.2	15.3 ± 1.5	8.5 ± 1.5	9.0 ± 1.5	15.5 ± 2.1	14.2 ± 1.9	10.2 ± 1.5

Table 5.6: Rate Pressure Product in Experimental Groups (mm Hg/min x 10^3). Values are mean ± SEM. * p <0.05 compared with control. S – stabilization, I – ischaemia, R – reperfusion, Wist - Wistar, GK - Goto Kakizaki, Cont - control, IPC - ischaemic preconditioning, LY - LY294002

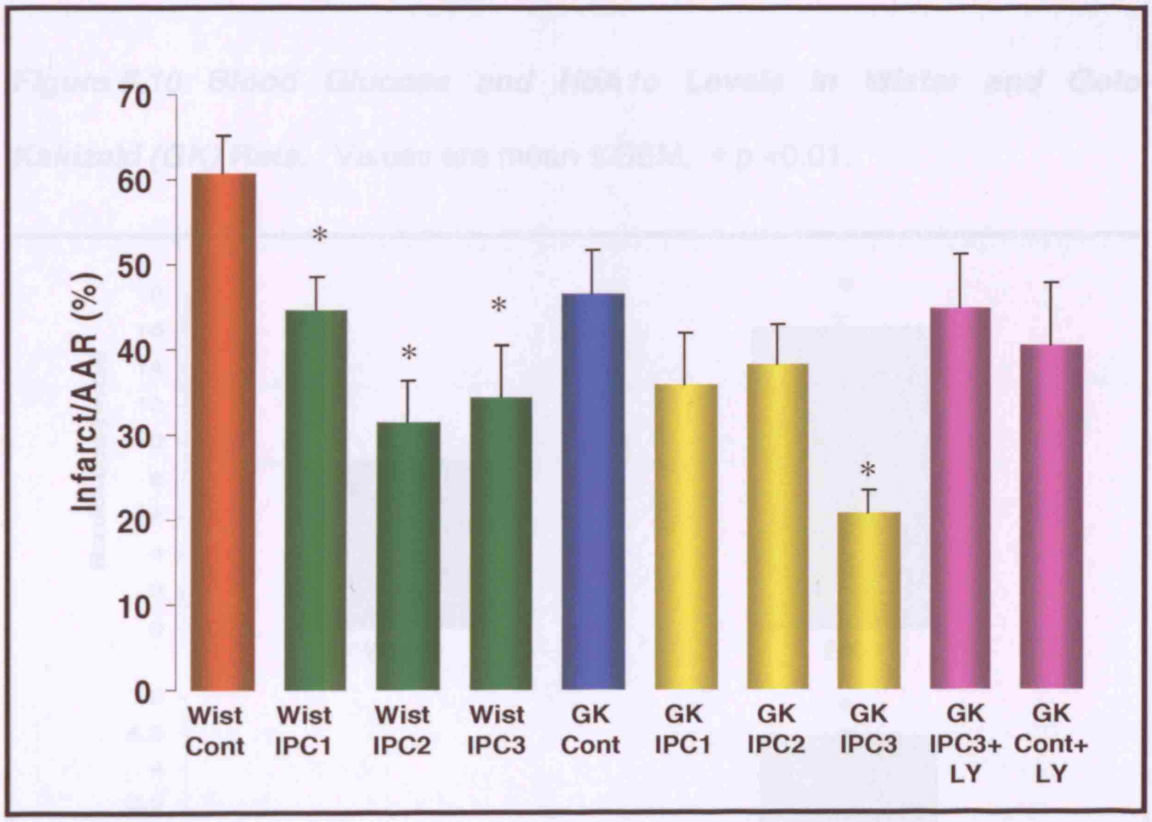
Group	S 10 min	S 30 min	I 5 min	I 30 min	R 5 min	R 30 min	R 120 min
Wist Cont	48.7 ± 4.2	41.1 ± 3.3	22.4 ± 2.5	27.5 ± 2.2	18.8 ± 2.8	23.9 ± 2.9	16.1 ± 3.3
Wist IPC1	51.6 ± 3.6	41.0 ± 2.7	21.9 ± 2.0	27.4 ± 3.1	23.8 ± 2.9	23.9 ± 2.9	17.2 ± 2.3
Wist IPC2	51.7 ± 2.7	43.4 ± 2.9	18.9 ± 2.2	21.4 ± 2.1	15.9 ± 1.9	19.9 ± 1.6	14.3 ± 1.6
Wist IPC3	40.6 ± 2.6	32.7 ± 3.8	15.2 ± 1.4*	15.7 ± 1.3*	14.9 ± 1.9	16.3 ± 1.5*	12.6 ± 1.0
GK Cont	39.3 ± 4.2	41.9 ± 5.0	21.9 ± 1.8	25.5 ± 2.5	22.2 ± 2.4	27.4 ± 3.0	18.0 ± 2.4
GK IPC1	40.4 ± 4.2	33.9 ± 4.9	19.1 ± 1.7	22.6 ± 2.4	20.9 ± 2.7	26.7 ± 3.7	19.6 ± 2.2
GK IPC2	41.6 ± 4.3	35.8 ± 5.1	17.6 ± 1.8	20.1 ± 1.8	20.7 ± 1.8	23.5 ± 1.4	18.1 ± 1.4
GK IPC3	38.3 ± 4.2	38.2 ± 4.1	16.9 ± 1.2	18.3 ± 1.4	18.9 ± 1.9	19.4 ± 1.8*	16.1 ± 1.2
GK IPC3+LY	39.9 ± 4.1	33.1 ± 3.6	14.9 ± 2.4*	17.5 ± 2.0	20.6 ± 2.5	22.7 ± 2.2	17.8 ± 2.0
GK Cont+LY	44.8 ± 3.9	33.2 ± 2.5	14.5 ± 4.3*	20.6 ± 6.0	23.7 ± 5.9	26.8 ± 5.0	20.8 ± 4.5

5.6.5.3 Infarct Size

The Threshold for Preconditioning is Elevated in the Diabetic Myocardium

In normal Wistar rats 1, 2, and 3 cycles of IPC significantly reduced infarct size represented as a percentage of the area at risk (AAR), compared with Wistar control hearts (from 60.7 ± 4.5% in Wist Cont to 44.7 ± 3.8% in Wist IPC1 to 31.4 ± 4.9% in Wist IPC2 to 34.3 ± 6.1% in Wist IPC3, $p<0.05$). However, in diabetic GK rats only 3 cycles of IPC reduced infarct size significantly compared with GK control hearts (from 46.6 ± 5.2% in GK Cont to 20.8 ± 2.6% in GK IPC3, $p<0.01$). Both 1 and 2 cycles of IPC failed to reduce infarct size significantly compared with GK control hearts (46.6 ± 5.2% GK Cont vs 35.8 ± 6.2% GK IPC1 vs 38.5 ± 4.5% GK IPC2, p=NS). However, the infarct reduction afforded by 3 cycles of IPC in the GK rat was completely abolished in the presence of the PI3K inhibitor LY294002 administered 5 minutes prior to the IPC protocol until 5 minutes after (from 20.8 ± 2.6% GK IPC3 vs 44.9 ± 6.4% with GK IPC3+LY, $p<0.01$), suggesting that PI3K may be important as a trigger in IPC as shown in Figure 5.9. LY294002 alone did not influence infarct size in the GK control group (40.6 ± 7.2% GK Cont+LY).

Figure 5.9: The Threshold for Preconditioning is Elevated in the Type 2 Diabetic Myocardium. Graph showing the reduction in infarct size achieved by 1, 2 and 3 cycles of ischaemic preconditioning (IPC) in Wistar (Wist) hearts. In the diabetic Goto-Kakizaki (GK) rat 1 and 2 cycles of IPC did not reduce infarct size significantly. Only 3 cycles of IPC was sufficient stimulus, to achieve a significant reduction in infarct size in diabetic GK hearts. Pharmacologically inhibiting PI3K during IPC, using LY294002 (LY), abolished the protection associated with 3 cycles of IPC in GK hearts. Values are mean ± SEM. * p <0.05.



5.6.5.4 *Blood glucose and glycated haemoglobin (HbA1c)*

Diabetic GK rats were characterized by significantly higher levels of blood glucose (16.1 mmol/l ± 0.6 in GK rats vs 9.0 mmol/l ± 0.3 in Wistar rats; $p<0.01$) and HbA1c (4.4% ± 0.1 in GK rats vs 3.0% ± 0.04 in Wistar rats; $p<0.01$) when compared to normal Wistar rats (see Figure 5.10). HbA1c values were considered diabetic when >3.45% using our assay. Regression analysis however, did not demonstrate any correlation between infarct size and blood glucose ($R^2=0.003$) or HbA1c ($R^2=0.163$) as shown in Figure 5.11 A+B.

Figure 5.10: Blood Glucose and HbA1c Levels in Wistar and Goto-Kakizaki (GK) Rats. Values are mean ± SEM. * $p<0.01$.

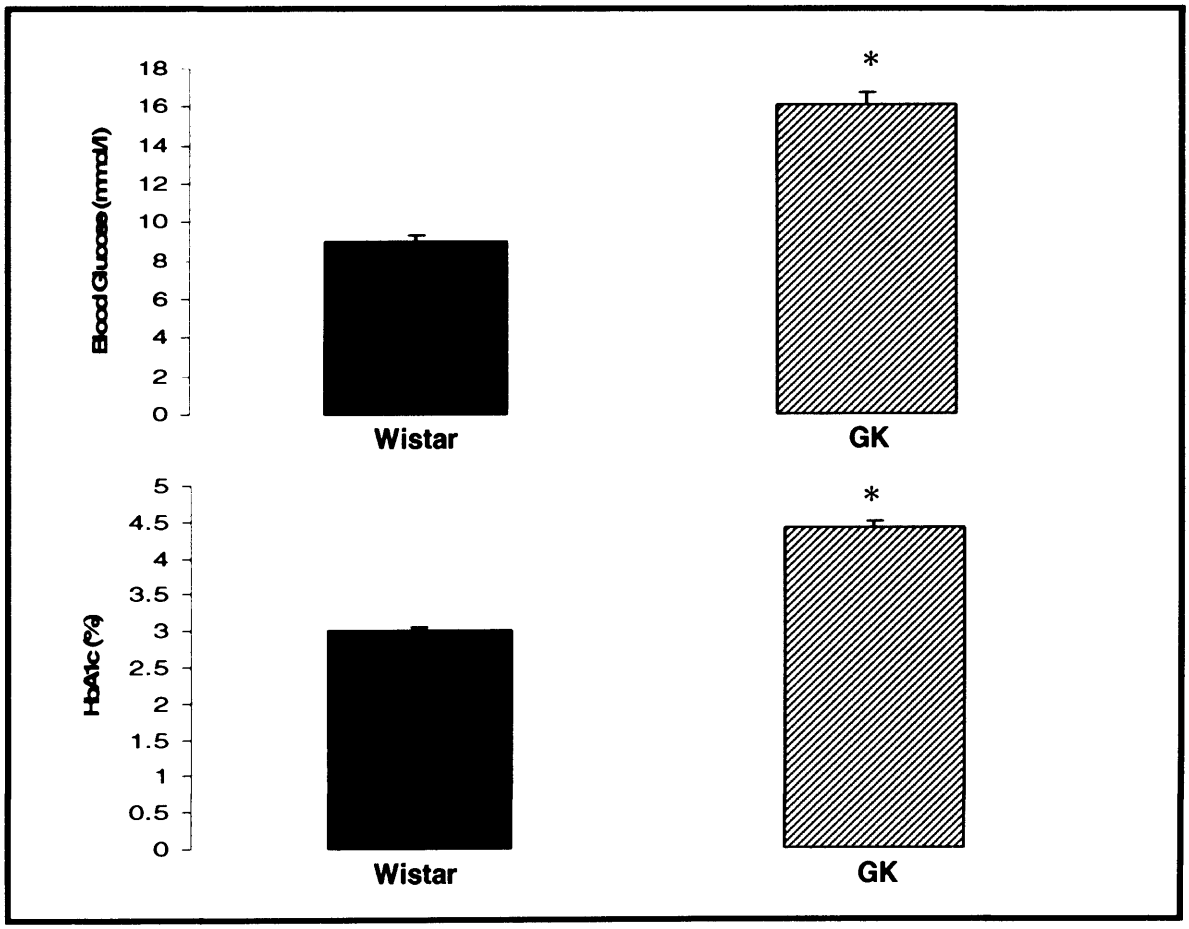
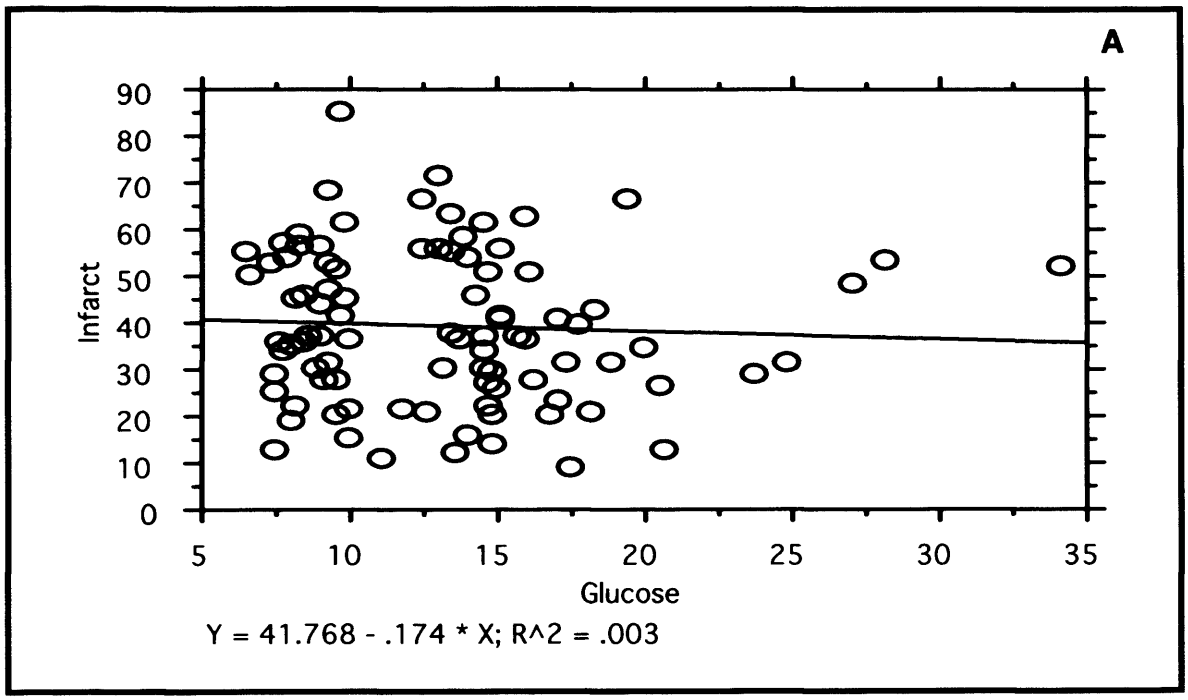
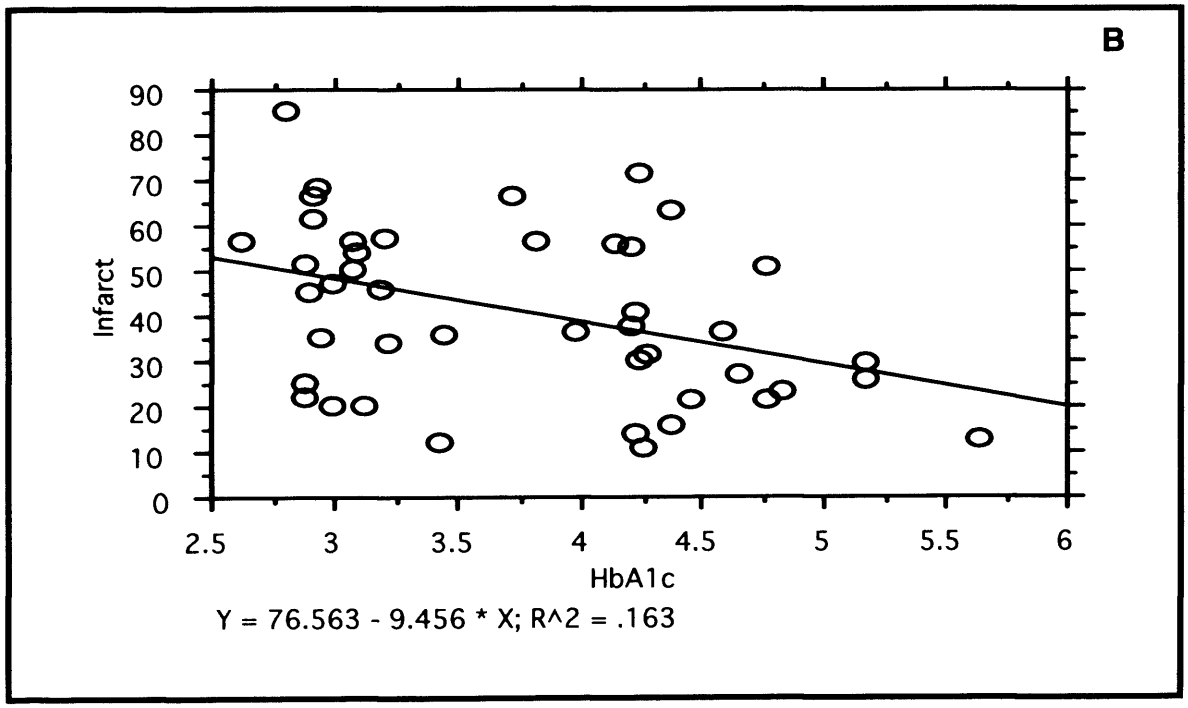


Figure 5.11: Blood Glucose and HbA1c Correlation with Infarct Size in Diabetic Hearts. Regression analysis did not demonstrate any correlation between infarct size and blood glucose (mmol/l) or HbA1c (%) levels.





5.7 Objective Two

To determine the role of the PI3K-Akt pathway in ischaemic preconditioning in type 2 diabetic rat hearts.

5.7.1 Animals

Male Wistar rats (300 to 400g, n=15) and male Goto-Kakizaki rats (300 to 400g, n=25) were used for this part of the investigation. The hearts were excised and Langendorff perfused as described in section 3.2.1. All hearts were subjected to protocols as described in section 5.7.2 below.

5.7.2 Experimental Protocols

The experimental protocols used in this part of the study are depicted in Figure 5.12. Hearts were randomly assigned to one of the following groups:

1) **Wistar Control** (n=5) hearts with 0.02% DMSO vehicle given alone during stabilization;

2) **Wistar IPC1** (n=5) comprising 5 minutes global ischaemia followed by 10 minutes reperfusion;

3) **Wistar IPC3** (n=5) comprising 3 cycles of 5 minutes global ischaemia followed by 10 minutes reperfusion;

4) **GK Control** (n=5) hearts with 0.02% DMSO vehicle given alone during stabilization;

5) **GK IPC1** (n=5) comprising 5 minutes global ischaemia followed by 10 minutes reperfusion;

6) **GK IPC3** (n=5) comprising 3 cycles of 5 minutes global ischaemia followed by 10 minutes reperfusion;

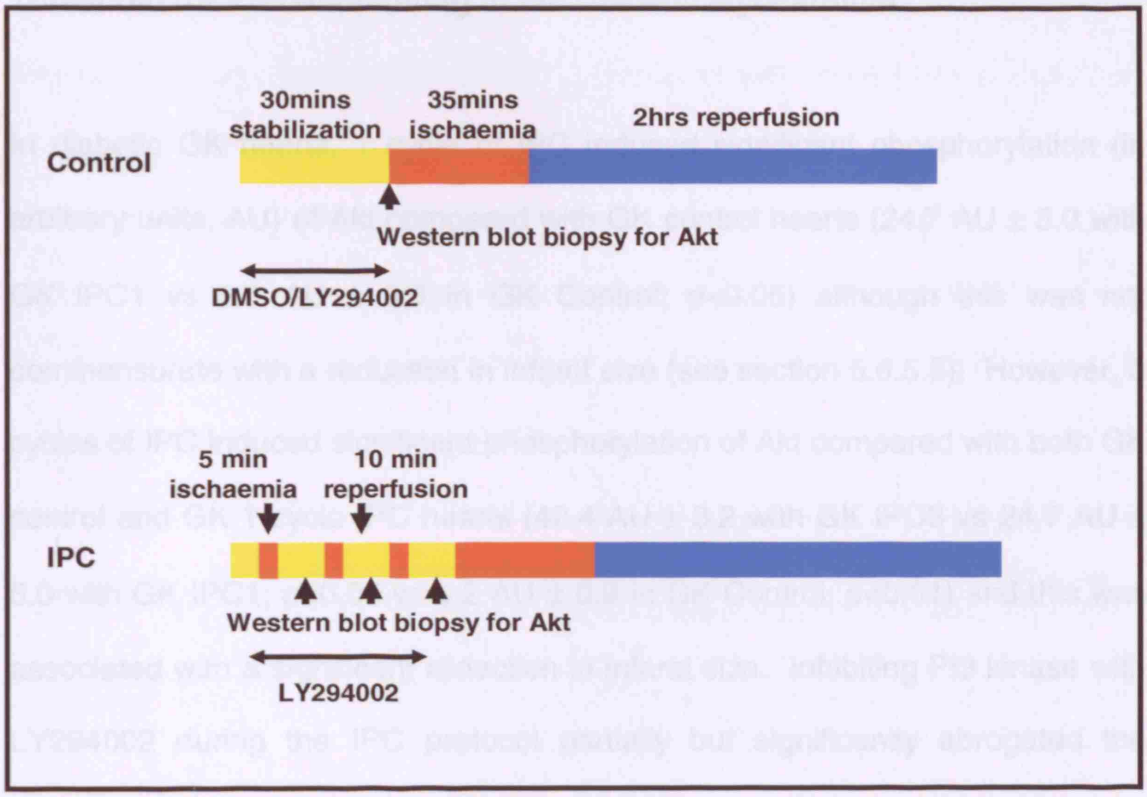
7) **GK IPC3+LY** (n=5), hearts were given the PI3K inhibitor LY294002 (LY, 15μmol/l) starting 5 minutes before and throughout the 3 cycle IPC protocol with a 5 minute wash-out;

8) **GK Control+LY** (n=5), hearts with LY294002 given alone during stabilization.

5.7.3 Measurement of Akt Phosphorylation Using Western Blot Analysis

Myocardial samples were taken for Western blot analysis at the end of stabilization or 5 minutes after the last IPC cycle from hearts that underwent the protocols as described in section 5.7.2. Western blot analysis was performed according to the methods described in section 3.3. The phosphorylation state of Akt (phospho-Akt, Ser 473) and total levels of Akt protein were analyzed, using antibodies obtained from Cell Signalling Technology (New England Biolabs). Levels of phosphorylated proteins were normalized to their total protein levels.

Figure 5.12: Experimental Protocols for Investigating the Role of Akt in Ischaemic Preconditioning in Type 2 Diabetic Hearts. IPC – ischaemic preconditioning, DMSO - dimethylsulphoxide



5.7.4 Results

5.7.4.1 Exclusions

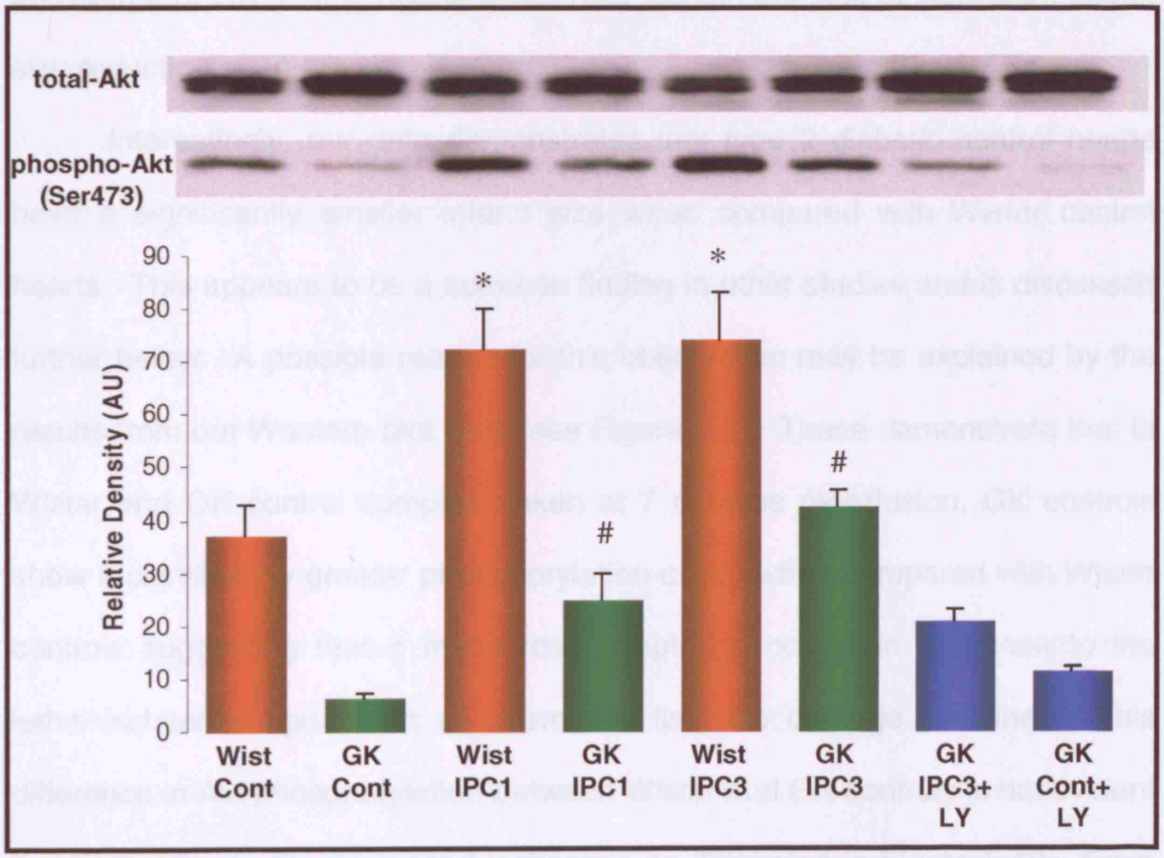
A total of 40 animals were used for this part of the study of which none were excluded.

5.7.4.2 Western Blot Analysis

Impaired Akt Phosphorylation Appears Responsible for the Elevated Threshold for Preconditioning in the Diabetic Myocardium

In diabetic GK hearts, 1 cycle of IPC induced significant phosphorylation (in arbitrary units, AU) of Akt compared with GK control hearts (24.7 AU ± 5.0 with GK IPC1 vs 6.2 AU ± 0.9 in GK Control; $p<0.05$) although this was not commensurate with a reduction in infarct size (see section 5.6.5.3). However, 3 cycles of IPC induced significant phosphorylation of Akt compared with both GK control and GK 1 cycle IPC hearts (42.4 AU ± 3.2 with GK IPC3 vs 24.7 AU ± 5.0 with GK IPC1; $p<0.05$ vs 6.2 AU ± 0.9 in GK Control; $p<0.01$) and this was associated with a significant reduction in infarct size. Inhibiting PI3 kinase with LY294002 during the IPC protocol partially but significantly abrogated the phosphorylation of Akt in GK IPC3 to levels similar to that seen with 1 cycle IPC (42.4 AU ± 3.2 with GK IPC3 vs 20.8 AU ± 2.1 with GK IPC3+LY; $p<0.01$) suggesting that Akt plays an important role in preconditioning. In normal Wistar rats 1 and 3 cycles of IPC led to significant phosphorylation of Akt compared with Wistar control hearts (37.1 AU ± 5.7 in Wistar Control vs 72.4 AU ± 7.5 in Wistar IPC1; $p<0.01$ vs 74.2 AU ± 9.1 in Wistar IPC3; $p<0.01$) (see Figure 5.13).

Figure 5.13: Western Blots of Ischaemic Preconditioning in Type 2 Diabetic Hearts. Representative Western blots demonstrating phosphorylated and total Akt levels. IPC induced phosphorylation of Akt in both Wistar (Wist) and diabetic GK hearts, although the increase was more marked in the Wistar hearts. In Wistar hearts both 1 and 3 cycles of IPC resulted in a significant increase in Akt phosphorylation. Although 1 cycle of IPC resulted in significant phosphorylation of Akt in GK hearts, this was not sufficient to reduce infarct size. Only 3 cycles of IPC produced significant phosphorylation of Akt that was commensurate with a significant reduction in infarct size in an LY294002 (LY) sensitive manner (*p<0.05, #p<0.05).



5.7.5 Discussion

The findings from the first section of this chapter demonstrate for the first time that the phenomenon of ischaemic **post**conditioning does not protect the type 2 diabetic myocardium from the effects of ischaemic-reperfusion injury unlike non-diabetic hearts as seen in Chapter 4. Whether this strategy is a "healthy" heart phenomenon i.e. is only effective in non-diseased hearts, requires further investigation by future studies. Western blot analysis of ischaemic postconditioning in the diabetic myocardium shows that this phenomenon fails to result in a significant phosphorylation of Akt over and above that of GK control hearts which may explain the lack of significant infarct size reduction seen.

Interestingly, our data demonstrates that type 2 diabetic control hearts have a significantly smaller infarct size when compared with Wistar control hearts. This appears to be a common finding in other studies and is discussed further below. A possible reason for this observation may be explained by the results from our Western blot data (see Figure 5.6). These demonstrate that in Wistar and GK control samples, taken at 7 minutes reperfusion, GK controls show a significantly greater phosphorylation of Akt when compared with Wistar controls suggesting that a myocardial adaptation occurs in response to the lethal ischaemic episode in an attempt to limit the damage sustained. This difference in Akt phosphorylation between Wistar and GK controls is not evident however **prior** to the prolonged ischaemia as illustrated in Figure 5.13. From Western blot control samples taken at the end of stabilization, the reverse is actually seen, that is, GK control hearts have a significantly lower level of

phosphorylated Akt than Wistar controls. Taken together, this suggests that the lethal ischaemic insult acts as a stimulus to trigger a pro-survival adaptive reponse to limit ischaemic-reperfusion injury in the diabetic myocardium resulting in smaller infarcts. The exact mechanisms responsible for this observation need further clarification.

We also report for the first time that the type 2 diabetic GK myocardium can benefit from the cardio-protective effects of ischaemic preconditioning, provided that the preconditioning stimulus is increased to reach the threshold necessary to achieve myocardial protection. In our study, this threshold required a preconditioning stimulus of 3 cycles of 5 minutes ischaemia and 10 minutes reperfusion prior to the prolonged ischaemic insult. It appears that impairment of pro-survival signalling cascades may be responsible for this elevated threshold. We demonstrate that although 1 cycle of IPC induced a significant phosphorylation of Akt, this did not result in a reduction of infarct size. However, 3 cycles of IPC in the diabetic heart resulted in a significantly greater phosphorylation of Akt than 1 cycle and this was commensurate with a significant reduction in infarct size. Furthermore, Western blot analysis did not demonstrate any significant differences in the total levels of Akt protein in diabetic GK hearts in all groups, indicating that the impairment in phosphorylation of Akt was not a result of lower levels of total Akt in diabetic hearts, but in the signalling process leading to Akt activation. Whether this impairment in Akt activation is due to abnormalities in upstream mediators of the Akt pathway for example PI3 kinase or PIP3 dependent kinase (PDK1) remains to be elucidated. However, it should be noted that our data does not prove a direct causal relationship between impaired Akt phosphorylation and

the elevated threshold for protection. The use of specific Akt inhibitors would help to clarify Akt's role in protection in this setting.

Interestingly, our present study supports the findings of Kondo and colleagues[161] who demonstrated that different defects in components of cell survival kinase cascades in diabetic models are not species specific but organ specific within the same species. In both type 1 and 2 diabetic mice, they showed that in the retina, the reduction in the phosphorylation of PDK1 and Akt was due to reduced total levels of PDK1 and Akt when compared with controls after insulin stimulation. However, within the same mice, the total levels of PDK1 and Akt were the same in the liver and the reduction in PDK1 and Akt phosphorylation was due to impaired activation of these proteins.

Evidence suggests that other components of cellular pro-survival pathways are defective in diabetic tissues as well as the heart. Hyperglycaemia has been shown to inhibit the pro-survival effect of vascular endothelial growth factor, leading to retinal cell apoptosis via tyrosine nitration of PI3 kinase which results in Akt inactivation and increased p38 MAP kinase activation[162]. Similar results have been demonstrated in rat hearts exposed to hyperglycaemic conditions which led to tyrosine nitration and apoptosis through the action of iNOS and NO release[163]. To our knowledge, our data demonstrates for the first time, that it is the impairment in cellular signalling cascades which is responsible for the inconsistent results reported in previous diabetic preconditioning experiments.

The lack of correlation between either glucose or HbA1c levels, or infarct size in our study, suggests that the severity of diabetes does not predict the

extent of myocardial infarction, but rather once a minimum level of diabetes is reached, the threshold for preconditioning is elevated.

Until recently, most animal studies have focused on ischaemic preconditioning in the diabetic heart using chemically induced type 1 diabetic animal models[145-148;150]. However, the conflicting data obtained from these studies should be interpreted with caution. Many of the animals died as a result of the chemical induction of diabetes and others displayed characteristics of stress probably as a consequence of the toxicity of the drugs (alloxan or streptozotocin) used. The non-specific effects of these drugs on the myocardium are not well known. In addition, these animal models of diabetes simulate type 1 diabetes[164], which in the clinical scenario, is the least common form in the human population. In this regard, chemically induced models of diabetes do not represent the most appropriate model in which to study the effects of myocardial protection. Animal models of type 2 diabetes should be used, as it is this form of diabetes which is prevalent worldwide and is associated with increased cardiovascular risk.

The Goto-Kakizaki rat is a selectively in-bred model of type 2 diabetes developed from the Wistar rat which has many similarities to the human form of the disease[158;159], and has been used extensively as a type 2 diabetic research model[160]. This appears to be a more appropriate diabetic research model since worldwide the most common form of diabetes is type 2 diabetes and its prevalence is steadily increasing[165]. To date, only one study has addressed the issue of preconditioning in a model of type 2 diabetes using an obese and lean animal. In that study, Kristiansen et al[149] used the GK rat to address the issue of ischaemic preconditioning and demonstrated that in their model, the diabetic

heart failed to show any reduction in infarct size when subjected to an IPC protocol of 4 cycles of 2 minutes ischaemia followed by 3 minutes reperfusion, a preconditioning stimulus which we would argue was insufficient to reach the threshold necessary to activate cardio-protective mechanisms in the type 2 diabetic heart. Furthermore, this study did not provide any mechanistic data to explain the lack of protection afforded by IPC in their model. In contrast to their study, the diabetic hearts in our study were able to be protected by IPC, although the IPC stimulus required was elevated. This would suggest that provided the IPC stimulus is sufficient, the diabetic heart can be protected from ischaemic-reperfusion injury.

Interestingly, our data supports the observations made by Kristiansen and colleagues[149] with respect to the smaller infarct size in diabetic animal hearts compared to non-diabetic hearts, following an episode of ischaemia-reperfusion. This appears to be a common finding in studies which have compared the myocardial infarct size in diabetic animal models with "normal" hearts[145;149;166] and suggests that in diabetic hearts, a myocardial adaptation occurs **after** a prolonged ischaemic insult which attempts to limit the damage sustained from the ischaemic injury, as mentioned above. However, the exact mechanisms responsible for this adaptation have yet to be elucidated. In our study, the Wistar control animals demonstrated larger infarct sizes compared to the diabetic GK animals, raising an important question as to whether a "normal" Wistar heart is the appropriate control with which to compare the type 2 diabetic GK hearts. Goto-Kakizaki rats have been developed by selective in-breeding of glucose intolerant Wistar rats since 1975[158], resulting in a species with many biochemical and metabolic similarities to human type 2 diabetes. Therefore, as

in clinical studies, a more appropriate control to compare a treatment in diabetic populations would be a non-treated diabetic rather than a "healthy" species. Nevertheless, this should not detract from the fact that the most important findings from our study, namely the requirement for a sufficient preconditioning stimulus to induce protection was observed within the diabetic Goto-Kakizaki group.

Since preconditioning can be achieved despite a raised preconditioning threshold, it is important to realise that failure of ischaemic **post**conditioning in type 2 diabetic hearts in this study may be the result of an insufficient postconditioning stimulus inadequate to trigger a protective signal, rather than failure of the strategy itself. Another important aspect is the fact that diabetic hearts appear to exhibit smaller infarcts overall and that the maximal effect of any infarct sparing intervention applied using standard protocols, may be too small to uncover any statistical difference. In order to reveal any significant benefit it may be necessary to increase postconditioning's maximal effect by increasing the protective stimulus.

In conclusion, we report for the first time that the type 2 diabetic myocardium cannot be protected by ischaemic postconditioning using our protocols but can be protected by ischaemic preconditioning. However, the threshold required to achieve this protection is elevated compared to non-diabetic hearts. We find that this elevation in threshold may be the result of impaired pro-survival pathways important in myocardial protection.

The findings from this study suggest that the human diabetic population may be more resistant to the protective effects of IPC, but that provided the preconditioning stimulus is sufficient, the diabetic myocardium can be protected.

One intriguing aspect would be to investigate whether this resistance to IPC-induced protection is reversed in diabetic species treated with either insulin or oral hypoglycaemic drugs.

Chapter Six: CONCLUSION

6.1 Summary of Study Findings

This thesis investigated the cardioprotective phenomenon of ischaemic postconditioning in both normal and type 2 diabetic hearts. In addition, the well established protective strategy of ischaemic preconditioning was investigated in type 2 diabetic hearts.

In Chapter 4, we demonstrated that ischaemic postconditioning reduced myocardial infarct size in isolated perfused rat hearts. We showed that postconditioning was effective in an in vitro animal model using a blood-free perfusate. This suggests that ischaemic postconditioning is mediated by mechanisms other than blood constituents as had been previously postulated[110]. To this effect, our data demonstrates for the first time that the effects of ischaemic postconditioning are mediated via the PI3K-Akt pro-survival pathway and its downstream targets, eNOS and p70S6K. Similarly, the pro-survival MEK1/2-ERK pathway has also been implicated to play a role in ischaemic postconditioning[113;133]. The implication of pro-survival cascades suggests that ischaemic postconditioning may protect the myocardium by the recruitment of the Reperfusion Injury Salvage Kinase (RISK) pathway in the early moments of reperfusion.

To date, the cardioprotective effects of ischaemic postconditioning had only been studied in normal animal hearts. In Chapter 5 we investigated the effects of ischaemic postconditioning and preconditioning in type 2 diabetic rat hearts, a major risk factor for coronary heart disease. We demonstrated that

the damaging effects of ischaemic-reperfusion injury could not be reduced by ischaemic postconditioning in diabetic hearts and that this lack of infarct sparing ability appears to be due to an insufficient activation of Akt above that seen in diabetic control hearts using our postconditioning protocols. To address the conflicting data surrounding the effectiveness of ischaemic preconditioning in diabetic hearts, firstly by choosing an appropriate diabetic model, we present evidence that the diabetic myocardium can be protected. The findings from our data shows that it is necessary to apply 3 cycles of IPC in order to achieve a significant reduction of myocardial injury suggesting that the IPC stimulus needs to be great enough to reach the threshold necessary to execute the protective signals. Once this threshold is achieved, a critical level of Akt phosphorylation occurs which translates into a reduction in infarct size. This study demonstrates for the first time that it may be the impairment of cellular signalling cascades that is responsible for the conflicting data seen regarding diabetic preconditioning.

6.2 Clinical Implications

An important finding from this study is that ischaemic postconditioning is a powerful cardioprotective strategy that exerts its beneficial effects within the immediate period surrounding the re-establishment of coronary reflow i.e. reperfusion. These effects appear to be mediated by recruitment of pro-survival kinases that comprise the RISK pathway which can also be activated by pharmacological agents and these may therefore be considered as postconditioning mimetics. Since the onset of reperfusion is more predictable

clinically with the use of thrombolytic drugs and percutaneous coronary intervention, the first few minutes of reperfusion provide an attractive window of opportunity to apply pharmacological postconditioning agents to current reperfusion strategies in order to reap a synergistic benefit in terms of myocardial salvage in those patients presenting with a myocardial infarction. An example of this was seen in the recently reported CREATE-ECLA trial[167], where the administration of glucose insulin potassium (GIK) solution failed to show any mortality benefit compared with current reperfusion strategies alone. However, it must be noted that in the majority of patients, GIK was administered many minutes after the onset of reperfusion and therefore the window of opportunity for cardioprotection may have been missed. Only 2.8% of patients were randomized within an hour which the scientific evidence would suggest is the important window.

With regards to the human diabetic population, our study suggests that this population of patients with a major risk factor for coronary heart disease may be more resistant to the beneficial effects of IPC, but that provided the preconditioning stimulus is sufficient, the diabetic myocardium can be protected. Whether this resistance is the direct result of the diabetic state has yet to be established but a very important issue to be addressed is whether pharmacological control of diabetes leads to lowering of this resistance to a level seen in non-diabetic hearts. This obviously has extremely important clinical implications.

6.3 Study Limitations

The data from this study was obtained using a Langendorff in vitro model however, as in most in vitro studies, further support for the conclusions drawn would have been achieved if this work could have been reproduced in a whole body in vivo model had time allowed. Nonetheless, important observations have been obtained from this thesis which should highlight important areas for further study.

One interesting aspect to address is the concentration of glucose in the modified Krebs-Henseleit buffer used in the experiments particularly concerning the diabetic data. Although the glucose concentration in our buffer is 11 mmol/l, which is high compared with in vivo values, this buffer has been used in large numbers of studies without cause for concern. However, it would be important to determine whether a lower glucose concentration, for example 5 mmol/l, would have any significant difference to the results. This is unlikely since the metabolic and detrimental effects of human diabetes are largely due to the effects of chronic high glucose levels and the glycosylation of functional proteins rather than acute glucose levels.

As discussed in section 5.7.5 one should be aware that failure of ischaemic postconditioning to limit myocardial infarction in type 2 diabetic hearts may not be due to the failure of the strategy itself. Since in our study, diabetic hearts have smaller infarct sizes, the maximal infarct sparing effect of our standard postconditioning protocol may not be powerful enough to uncover a significant difference compared with control hearts. Increasing the duration of cycles or number of cycles may possibly raise the potency of protection to

reveal a significant difference. This should form the basis of further studies because increasing the preconditioning stimulus greater than that used in standard protocols results in a limitation of myocardial injury not otherwise seen.

The implication that impairment of Akt phosphorylation in diabetic hearts is responsible for the elevated threshold necessary to achieve preconditioning-induced cardioprotection should be taken with caution. Our study does not demonstrate a direct causal relationship between Akt and the threshold but merely indicates that it appears to be an impairment of pro-survival signalling that is responsible. The exact components of these pathways responsible has yet to be elucidated. However, our results show that Akt via PI3K may be a common downstream target that is affected in this impairment. The use of specific kinase inhibitors would help to determine those which may be defective or important in these pro-survival cascades.

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